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in Mammary Tumorigenesis

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The aim of the studies covered under this report was to start addressing the potential role of protein tyrosine phosphatases in mammary tumorigenesis. On theoretical grounds, this class of enzymes could be expected to either act as negative regulators of growth (e.g. tumor suppressors), or to act oncogenically, e.g. by their ability to activate kinases that are negatively regulated by phosphorylation. We have addressed this question using RPTPa as a model PTP. Expression of this PTP has been surveyed in human breast cancers, in vitro studies have been performed to assess the effect of this PTP on growth properties and tumorigenicity of mammary carcinoma cells. Both approaches have led to the conclusion that increased expression of this PTP correlates with a less advanced stage of tumor progression. A number of studies have also been started to address the effect of RPTPa mouse gene ablation on models of mouse mammary tumorigenesis. These results suggest RPTPa may be useful as a marker for tumor progression, and raise a cautionary note regarding the use of PTP inhibitors in tumor therapy. These studies have been delayed with respect to the original timetable, but are proceeding.

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Oncogene, 19 (2000), 4979-4987.	
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Current Biology (1999), 505-511.	
3) A. Petrone and <u>J. Sap</u> : Emerging aspects of receptor protein tyrosine phosphatase function: lifting fog or simply shifting?	
Journal of Cell Science 113 (2000), 2345-2354.	

## **INTRODUCTION**

The possible role of protein tyrosine phosphatases (PTPs) in breast cancer has been poorly explored. Yet, there are abundant theoretical reasons that warrant such a line of investigation. While the scope of this question clearly exceeds by far the feasibility limits of one grant, we chose RPTP $\alpha$ , which is widely expressed and one of the better understood PTPs, as a model. It had previously been demonstrated that RPTP $\alpha$  is capable of activating SRC family kinases (SFKs), and of potentiating signaling through the EGF-receptor. We hypothesized that RPTP $\alpha$  expression might be selected for and potentiate or mimic the HER2/neu activation. In this proposal, we wanted to test this hypothesis by 1) analyzing the correlation between RPTP $\alpha$  expression and other tumor markers directly in human tumor samples; 2) performing *in vitro* studies on the effect of RPTP $\alpha$  on cell growth parameters; and 3) studying the effect of RPTP $\alpha$  on tumor development in animals.

## **BODY**

### **Aim 1: Analysis of the expression of RPTP $\alpha$ in human breast cancer.**

To explore its potential contribution to human neoplasia, we surveyed RPTP $\alpha$  protein levels in primary human breast cancer. We found RPTP $\alpha$  levels to vary widely among tumors, with 29 % of cases manifesting significant overexpression. High RPTP $\alpha$  protein levels correlated significantly with low tumor grade and positive estrogen receptor status. To our knowledge, this is the first example of a study correlating expression level of a specific *bona fide* PTP with neoplastic disease status in humans. These data are presented in a published manuscript (Ardini et al., 2000; see appendix).

### **Aim 2: In vitro studies on the effect of RPTP $\alpha$ on cell growth and transformation parameters**

- a) RPTP $\alpha$  was expressed in MCF-7 breast carcinoma cells. We have successfully demonstrated that this caused growth inhibition (which correlated with the level of RPTP $\alpha$  overexpression), and increased accumulation in G<sub>0</sub> and G<sub>1</sub>. Biochemical characterization revealed that RPTP $\alpha$  expression caused increased kinase activity of endogenous c-SRC. Furthermore, G<sub>1</sub> arrest associated with RPTP $\alpha$  expression in MCF7 cells is accompanied by a drastic increase in p27 expression. These findings were surprising, and contrast with claims in the literature that RPTP $\alpha$  expression can be transforming. The implications of these findings are discussed in the conclusions section.
- b) The above results were obtained using serum. We have been less successful in performing analogous studies on the effect of defined growth factors on cell proliferation. However, we do continue to explore the effect of RPTP $\alpha$  on growth factor responses that do not involve proliferation, such as the differentiative response. We have obtained interesting data in this respect (Yang et al., submitted), and intend to revisit this issue in mammary cells.
- c) In an indirectly related series of experiments, using RPTP $\alpha$ -/- fibroblasts, we have obtained evidence for a role for RPTP $\alpha$  in integrin-dependent events mediated by SRC. Particularly, in fibroblasts, RPTP $\alpha$  appears involved in integrin-mediated phosphorylation of p130cas and FAK, and ERK activation (Su et al., 1999; see appendix). While not part of the original plan of experiments planned under this grant, these results nevertheless provide valuable insights that we will use for further analysis (see recommended changes for further work below).



### Aim 3: The effect of RPTP $\alpha$ on tumor development in animals

- a) We generated N202 mouse cell lines expressing RPTP $\alpha$  (and parallel negative controls). Upon inoculation into nude mice, we observed a significant inhibition of two parameters: tumor growth (upon subcutaneous injection) and metastasis (to the lung, upon intravenous injection).
- b) We have attempted to generate transgenic lines of RPTP $\alpha$  under the control of the MMTV LTR, in order to target its expression to the mammary epithelium, and investigate its effects on tumor induction. This approach has been unsuccessful. We have generated 3 founder lines, but were unable to detect RPTP $\alpha$  expression in any of them. We also did not observe any enhanced rate of spontaneous tumor induction.
- c) Rather than continue to attempt the same approach outlined in b) above, we have radically changed our approach. We generated a strain of RPTP $\alpha$  null mice in the lab (Su et al., 1999, appendix), and backcrossed this strain for 5 generations into the FVBN background. Moreover, we wished to take into account the fact that there is a PTP that is highly similar to RPTP $\alpha$ , i.e. RPTP $\epsilon$  (Elson et al., 1999), which has also been implicated in mammary tumor formation, and may have partially overlapping functions with RPTP $\alpha$ . We have embarked on a collaboration with the Elson lab (Weizmann Institute, Rehovot, Israel), and a double knock-out line of mice (i.e. for both RPTP $\alpha$  and RPTP $\epsilon$ ) has been generated. In this collaboration, these mice have been crossed with an existing strain of HER2 overexpressing mice. This means that we are now able to analyze the effect of the absence of RPTP $\alpha$  and RPTP $\epsilon$  on the kinetics of tumor induction by this oncogene. Monitoring of these mice for tumor incidence is now already underway. We are very excited about this approach, and expect it to provide valuable information on the role of RPTP $\alpha$  and  $\epsilon$  on tumor induction. Not unexpectedly, given the extensive crossing scheme that was required, this work is now extending beyond the original time-line of the grant. However, we do consider it an integral and active part of the present grant, and will hence acknowledge the DOD upon publication.
- d) N.B.: In generating and using these RPTP $\alpha$ <sup>-/-</sup> mice, we observed that RPTP $\alpha$ <sup>-/-</sup> females are virtually totally infertile. We are in the process of determining whether this may reflect abnormalities in steroid or pituitary hormones. This finding may or may not be relevant for breast cancer.

### List of personnel receiving pay from the research effort:

Jan Sap, Ph.D.  
Elena Ardini, Ph.D.  
Adina Dusa, M.D.

## **KEY RESEARCH ACCOMPLISHMENTS**

- a) Overexpression of RPTP $\alpha$  in human tumors is a common event. It is significantly correlated with lower-grade.
- b) Increased expression of RPTP $\alpha$  in MCF-7 cells results in reduced growth rates, and an enhanced accumulation in the G1 phase of the cell cycle associated with p27 accumulation.
- c) Forced expression of RPTP $\alpha$  in mouse mammary N202.1A cells results in reduced tumorigenicity in nude mice assays. This was documented by 2 parameters: tumor growth following subcutaneous injection, and metastasis formation following intravenous injection.

- d) An attempt has been made to generate MMTV-RPTP $\alpha$  transgenic mice. While 3 lines could be generated, none expressed at the protein level. However, as an alternative, RPTP $\alpha$ -/- mouse strains are being put to active use for further study in this field (crosses with RPTP $\epsilon$ -/- and with HER2 transgenics).

## **REPORTABLE OUTCOMES**

- 1) E. Ardini, L. Yang, S. Menard, and J. Sap (2000): Expression of receptor protein tyrosine phosphatase alpha (RPTP $\alpha$ ) in primary human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo* " Oncogene, 19 (2000), 4979-4987.  
(SEE APPENDIX)
- 2) J. Su, M. Muranjan, and J. Sap (1999): Receptor protein tyrosine phosphatase  $\alpha$  (RPTP $\alpha$ ) is an endogenous activator of Src family kinases, and controls integrin-mediated responses in fibroblasts.  
Current Biology, 505-511.  
(SEE APPENDIX)
- 3) A. Petrone and J. Sap (2000): Emerging aspects of receptor protein tyrosine phosphatase function: lifting fog or simply shifting? (*invited review*).  
Journal of Cell Science 113 (2000), 2345-2354.  
(SEE APPENDIX)
- 4) E. Ardini, R. Agresti, E. Tagliabue, M. Greco, P. Aiello, L-T. Yang, S. Menard, and J. Sap (1999): Expression of protein tyrosine phosphatase alpha (RPTP $\alpha$ ) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo*. Abstract presented at the Salk/Embl conference on Oncogenes and Growth control. The Salk Institute, San Diego, CA, August 18-22, 1999.
- 5) E. Ardini, R. Agresti, E. Tagliabue, M. Greco, P. Aiello, L-T. Yang, S. Menard, and J. Sap (2000): Expression of protein tyrosine phosphatase alpha (RPTP $\alpha$ ) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo*. Proceedings, Department of Defense Breast Cancer Research Program meeting, Atlanta, GA, June 8-11, 2000.
- 6) At least one additional publication is expected to result from the ongoing studies described in aims 3b and 3d.

## **CONCLUSIONS**

1. expression of RPTP $\alpha$  in primary human tumors correlates significantly with grade.
2. expression of RPTP $\alpha$  in cultured mammary tumor cell lines results in G1 arrest in vitro, and reduced tumor growth and metastasis in nude mice.
3. RPTP $\alpha$ -induced G1 arrest is accompanied by p27 induction
4. RPTP $\alpha$  expression in human tumors does not correlate with SRC kinase activity.
5. Mice lacking RPTP $\alpha$  are not prone to a significant increase in solid tumors

### **Associated recommendations for further work in this field:**

1. The basis of the observed transforming character of RPTP $\alpha$  in certain settings but not others is poorly understood. It may reflect cell type specificity (fibroblast vs. epithelial), or alternative splice variants.
2. There is a need to investigate whether the growth inhibitory function of RPTP $\alpha$  in mammary epithelial cells is a direct consequence of SRC activation
3. It will be very informative to determine whether RPTP $\alpha$  overexpression in tumors is an independent prognostic parameter, that can increase prognostic fidelity.

## **EVALUATION OF KNOWLEDGE AS A SCIENTIFIC OR MEDICAL PRODUCT:**

- 1) These studies establish RPTP $\alpha$  expression in breast tumors as a potential marker for limited tumor progression or/and improved prognosis.
- 2) PTPases, and particularly RPTP $\alpha$ , have often been viewed as promising targets for pharmacological inhibition in tumor therapy. Our data suggest this view may be premature, and needs to be explored further.

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Yang L.-T., Alexandropoulos, A., and Sap J.: Src-mediated neurite outgrowth occurs through recruitment of Crk to the p130cas-related docking protein Sin/Efs, and is independent of sustained ERK activation. *J. Biol. Chem.* (2001) *under revision*.

# Expression of protein tyrosine phosphatase alpha (RPTP $\alpha$ ) in human breast cancer correlates with low tumor grade, and inhibits tumor cell growth *in vitro* and *in vivo*

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Tyrosine phosphorylation is controlled by a balance of tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Whereas the contribution of PTKs to breast tumorigenesis is the subject of intense scrutiny, the potential role of PTPs is poorly known. RPTP $\alpha$  is implicated in the activation of Src family kinases, and regulation of integrin signaling, cell adhesion, and growth factor responsiveness. To explore its potential contribution to human neoplasia, we surveyed RPTP $\alpha$  protein levels in primary human breast cancer. We found RPTP $\alpha$  levels to vary widely among tumors, with 29% of cases manifesting significant overexpression. High RPTP $\alpha$  protein levels correlated significantly with low tumor grade and positive estrogen receptor status. Expression of RPTP $\alpha$  in breast carcinoma cells led to growth inhibition, associated with increased accumulation in G<sub>0</sub> and G<sub>1</sub>, and delayed tumor growth and metastasis. To our knowledge, this is the first example of a study correlating expression level of a specific *bona fide* PTP with neoplastic disease status in humans. *Oncogene* (2000) 19, 4979–4987.

**Keywords:** tyrosine phosphatase; c-Src; PTP; breast cancer; tumor marker

## Introduction

The crucial role of tyrosine phosphorylation in control of cell growth, motility, and invasiveness is reflected in the contribution of tyrosine kinases to neoplastic transformation (Porter and Vaillancourt, 1998). In breast cancer, HER2/neu overexpression is an important prognostic indicator, and constitutes a therapeutic target (Ross and Fletcher, 1999), but other receptor tyrosine kinases (RTKs) are also overexpressed (Barker *et al.*, 1995; Ellis *et al.*, 1998; Ghoussoub *et al.*, 1998; Maggiora *et al.*, 1998).

Increased activity of (non-receptor) Src-family kinases (SFKs) in neoplasia has been recognized for a long time (Maa *et al.*, 1995; Muthuswamy *et al.*, 1994; Ottenhoff-Kalff *et al.*, 1992). Firm genetic evidence implicating c-Src in human cancer was provided recently by the finding of a Src-activating point mutation in advanced colon tumors (Irby *et al.*,

1999). This somatic mutation introduces a stop codon immediately adjacent to Y530, which constitutes the main negative regulatory phosphorylation site of SFKs. Phosphorylated Y530 stabilizes an inactive conformation through intramolecular interaction with the c-Src SH2 domain (Schwartzberg, 1998). The mutation enhances c-Src kinase activity, and is transforming, tumorigenic, and promotes metastasis (Irby *et al.*, 1999), providing powerful evidence that selection for SFK activation contributes to tumor progression.

The biological activity of tyrosine kinases is highly context-dependent, and the identification of cellular modifiers of tyrosine-kinase initiated responses has therefore constituted a rewarding field of study. For instance, the signaling pathways downstream of HER2/neu are co-determined by its heterodimerization partners within the erbB family (Carraway and Cantley, 1994). Two membrane proteins were recently shown to modulate HER2/neu or EGF-receptor signaling (Carraway *et al.*, 1999; Kharitonov *et al.*, 1997).

The activity of SFKs is controlled by RTKs, the non-receptor kinase Csk, and protein tyrosine phosphatases (PTPs) (Schwartzberg, 1998). While altered expression or activity of PTPs might contribute to neoplasia, their role in, or behavior during, tumor progression is almost completely unstudied. The concept of clinically relevant, anti-oncogenically acting, PTPs has received support in the case of PTEN/MMAC, but the lipid phosphatase activity of the latter may be the actual mediator of its biological function (Machama and Dixon, 1999). However, PTPs can also act as positive regulators. They may increase substrate phosphorylation/dephosphorylation cycling rates (Fischer *et al.*, 1991). SHP-2 is required for the response to some mitogens (Feng, 1999). CD45, by acting as an activator of SFKs, is required for tyrosine phosphorylation and other signaling events upon T-cell receptor activation (Neel, 1997). However, inhibitory roles for SHP-2 and CD45 have also been described; the function of PTPs in growth control may thus be lineage- and signal-specific (Petrone and Sap, 2000).

PTPs constitute a large family of structurally diverse enzymes (Neel and Tonks, 1997). Only very few studies so far have addressed the possibility of deregulation of members of this family in breast cancer. Certain PTPs can inhibit growth of breast cancer cell lines (Keane *et al.*, 1996; Zhai *et al.*, 1995). PTP activity rises when breast cancer cells are growth inhibited by antiestro-

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gens (Freiss and Vignon, 1994). In primary human tumors, membrane PTP activity correlates with the presence of tumor positive axillary lymph nodes, whereas cytosolic activity correlates with the mitotic index (Ottenhoff-Kalff *et al.*, 1995). Increased PTP $\epsilon$  levels were observed in mouse tumors induced by HER2/neu or v-Ha-Ras, but not by *c-myc* or *int-2* (Elson and Leder, 1995b), and constitutive PTP $\epsilon$  expression increases the risk of mammary tumor development in transgenic mice (Elson, 1999).

In this study, we focused our attention on RPTP $\alpha$ . Three features of this receptor PTP suggest its potential relevance to oncogenic transformation. First, it is associated, via a tyrosine phosphorylation site in its C-terminus, with the Grb2 adaptor (den Hertog and Hunter, 1996; Su *et al.*, 1996), a regulator of Ras. We and others have shown that simultaneous association of Grb2 with the Ras activator Sos and with RPTP $\alpha$  is probably mutually exclusive, making it unlikely that RPTP $\alpha$  activates Ras by membrane recruitment of Sos (den Hertog *et al.*, 1994; Su *et al.*, 1996). However, Sos recruitment by Grb2 to RPTP $\alpha$  is not the only way in which RPTP $\alpha$  may affect Ras signaling; for instance, RPTP $\alpha$  may sequester Grb2 away from Sos; or Grb2 may recruit Grb2-bound docking proteins for dephosphorylation by RPTP $\alpha$ . Second, overexpression of RPTP $\alpha$  is observed in advanced human colon carcinoma (Tabiti *et al.*, 1995), and a closely related PTP, RPTP $\epsilon$ , is implicated in mouse mammary tumorigenesis (Elson and Leder, 1995b) (Elson, 1999). Third, RPTP $\alpha$  is an important regulator of SFK activity, by controlling dephosphorylation of the carboxy-terminal negative regulatory Y530 site of c-Src. Cells lacking RPTP $\alpha$  display reduced Fyn and c-Src kinase activities, and exhibit defects similar to those resulting from lack of c-Src (Ponniiah *et al.*, 1999; Su *et al.*, 1999). Conversely, RPTP $\alpha$  overexpression results in elevated c-Src kinase activity (den Hertog *et al.*, 1993; Zheng *et al.*, 1992, 2000). Intriguingly, activation of the c-Src kinase is often observed in breast and colon tumors (Cartwright *et al.*, 1990; Ottenhoff-Kalff *et al.*, 1992), and can potentiate signaling by the ErbB family (Tice *et al.*, 1999).

The properties of RPTP $\alpha$  thus led us to hypothesize that it could promote neoplastic transformation or progression. We were particularly guided by the hypothesis that increased RPTP $\alpha$  expression might occur in tumors as a result of selective pressure for enhanced c-Src kinase activity, and would correlate with more advanced disease. To test this issue, we analysed RPTP $\alpha$  expression in primary breast tumors, and the effect of its transfection on growth of breast carcinoma cell lines. Unexpectedly, we observed that overexpression of RPTP $\alpha$  is associated with lower grade, and while activating c-Src, acts in an anti-proliferative manner *in vitro* and *in vivo*.

## Results

### High levels of RPTP $\alpha$ protein in a subset of human breast tumors

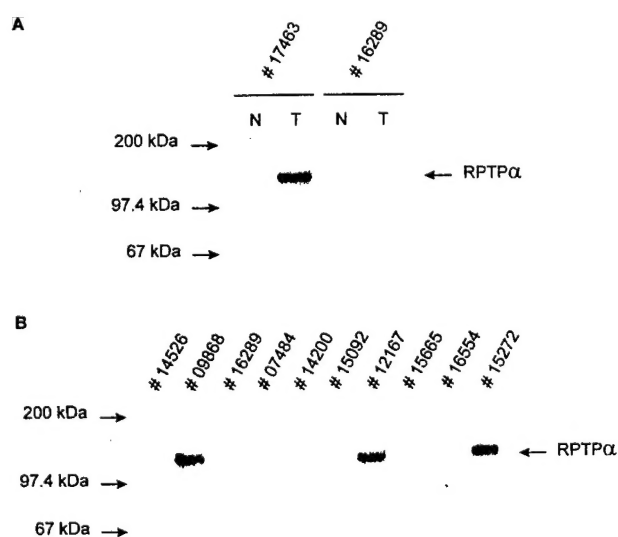
Cases of primary breast carcinoma were analysed for RPTP $\alpha$  expression by anti-RPTP $\alpha$  immunoblotting of protein lysates extracted from samples obtained at

surgery. This analysis revealed, in a subset of tumors, a single 130 kDa protein species reacting with an anti-RPTP $\alpha$  antiserum raised against the intracellular domain of the protein (Figure 1). In transient transfection experiments in 293 cells, this antibody had shown marginal cross-reactivity with RPTP $\epsilon$ , the PTP most closely related to RPTP $\alpha$  (data not shown). To confirm specificity, and to exclude cross-reaction with RPTP $\epsilon$ , samples were therefore also analysed by immunoprecipitation using antiserum 210, directed against an epitope which is not conserved between RPTP $\alpha$  and RPTP $\epsilon$ , followed by immunoblotting with antiserum 35, against the C-terminus of RPTP $\alpha$ . This experiment (not shown) confirmed that the protein species seen in Figure 1 corresponded to RPTP $\alpha$ . Analysis of a total of 51 cases revealed that, whereas RPTP $\alpha$  was only marginally detectable in the majority of tumors, its expression was substantially increased in 15 tumor samples out of the 51 analysed (29%). In the case of 13 patients, we were able to simultaneously analyse non-diseased tissue neighboring the tumor. While five out of the 13 corresponding tumors (38%) manifested increased RPTP $\alpha$  protein expression levels, all of the 13 matched normal neighboring tissue samples displayed a barely detectable background level of RPTP $\alpha$  expression (Figure 1 and data not shown).

### RPTP $\alpha$ expression correlates with low tumor grade

We subsequently analysed the correlation between RPTP $\alpha$  overexpression and pathological, clinical and biological parameters. No differences in histotype distribution were observed between non-expressing (5/36=14% lobular; 29/36=80% ductal) and RPTP $\alpha$  expressing (2/15=13% lobular; 11/15=73% ductal) carcinomas.

However, as shown in Table 1, high level expression of RPTP $\alpha$  was found significantly correlated with low



**Figure 1** Expression of RPTP $\alpha$  protein in human breast tumors. Total protein lysates from tumor or normal tissue from the same patient were subjected to anti-RPTP $\alpha$  immunoblotting, using an antiserum raised against the intracellular domain of RPTP $\alpha$ . A total of 51 tumors were analysed in this manner. (a) Two representative pairs of tumor (T) and same-patient neighboring normal tissue (N). (b) Nine more tumor samples; levels in control (non-diseased) tissue were virtually undetectable (not shown)



grade ( $P=0.02$ ) (Pereira *et al.*, 1995), and positive estrogen receptor status ( $P=0.04$ ). In addition, RPTP $\alpha$ -positive tumors tended to be HER2/neu-negative, to display lower proliferation indices, to be of smaller size, and to manifest a lower degree of lymph node involvement, although these distinctions were not at the level of statistical significance.

#### High RPTP $\alpha$ expression in human tumors is not due to gene amplification

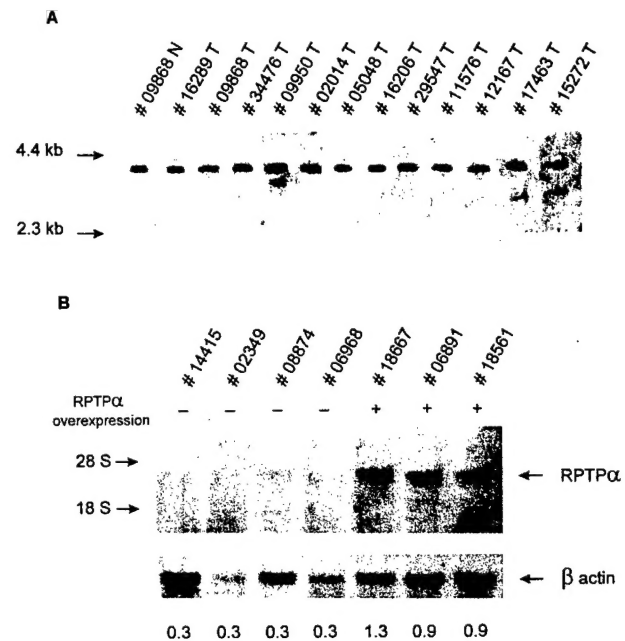
To investigate if overexpression of RPTP $\alpha$  was due to gene amplification, we performed Southern blot analysis on genomic DNA extracted from the primary tumors. Eleven cases that resulted positive for RPTP $\alpha$  overexpression (as revealed by immunoblotting), one case with no overexpression, and one normal mammary tissue sample were analysed using two different probes corresponding to the extracellular region of RPTP $\alpha$ , as well as using a  $\beta$ -actin probe (to normalize for loading). The results, shown in Figure 2a, indicate that none of the cases under study displayed RPTP $\alpha$  gene amplification.

#### Elevated RPTP $\alpha$ protein levels in human tumors correlate with enhanced mRNA levels

In the absence of amplification as a plausible mechanism (Figure 2a), we performed Northern analysis on a subset of the tumors analysed, to determine whether differences in RPTP $\alpha$  protein expression might reflect differences in mRNA content. In this experiment, we observed that three out of three tumors known to express high RPTP $\alpha$  protein levels (18667, 06891 and 18561; i.e. three right lanes) contained significantly higher level RPTP $\alpha$  mRNA levels than four cases that had tested undetectable for RPTP $\alpha$  protein (14415, 02349, 08874 and 06968; i.e. four left lanes) (Figure 2b). This result indicates that increased mRNA levels are the likely mechanism behind the enhanced RPTP $\alpha$  protein levels seen in primary tumors. Of note, enhanced RPTP $\alpha$  mRNA levels were also observed in a subset of primary human colon cancer cases (Tabiti *et al.*, 1995).

#### RPTP $\alpha$ overexpression in mammary cells leads to decreased growth capability

The effect of RPTP $\alpha$  on growth parameters was investigated in the human breast cancer cell line MCF7. In a first approach, an RPTP $\alpha$  expression vector (or the corresponding empty vector control) was transfected into MCF7 cells, and the number of



**Figure 2** (a) Southern blotting analysis of DNA from a set of human breast tumors (T) or one instance of neighboring normal tissue (N). All tumors overexpressed RPTP $\alpha$ , except 16289. Extracted DNA was digested with *EcoRI*, separated by gel electrophoresis, and analysed with a probe corresponding to the RPTP $\alpha$  extracellular domain. The slightly increased signal in lanes #09950T and 17463T could be entirely accounted for by increased gel loading, as established through rehybridization with a  $\beta$ -actin probe (data not shown). (b) Northern analysis on RPTP $\alpha$  mRNA from tumors showing high (18667, 06891, 18561) or low (14415, 02349, 08874, 06968) RPTP $\alpha$  protein levels. Twenty  $\mu$ g total RNA were loaded per lane and probed with a full-length RPTP $\alpha$  cDNA (top). Loading was normalized for by re-probing with a  $\beta$ -actin probe (bottom); numbers indicate RPTP $\alpha$ /beta-actin signal ratios for each lane, as determined by densitometric scanning

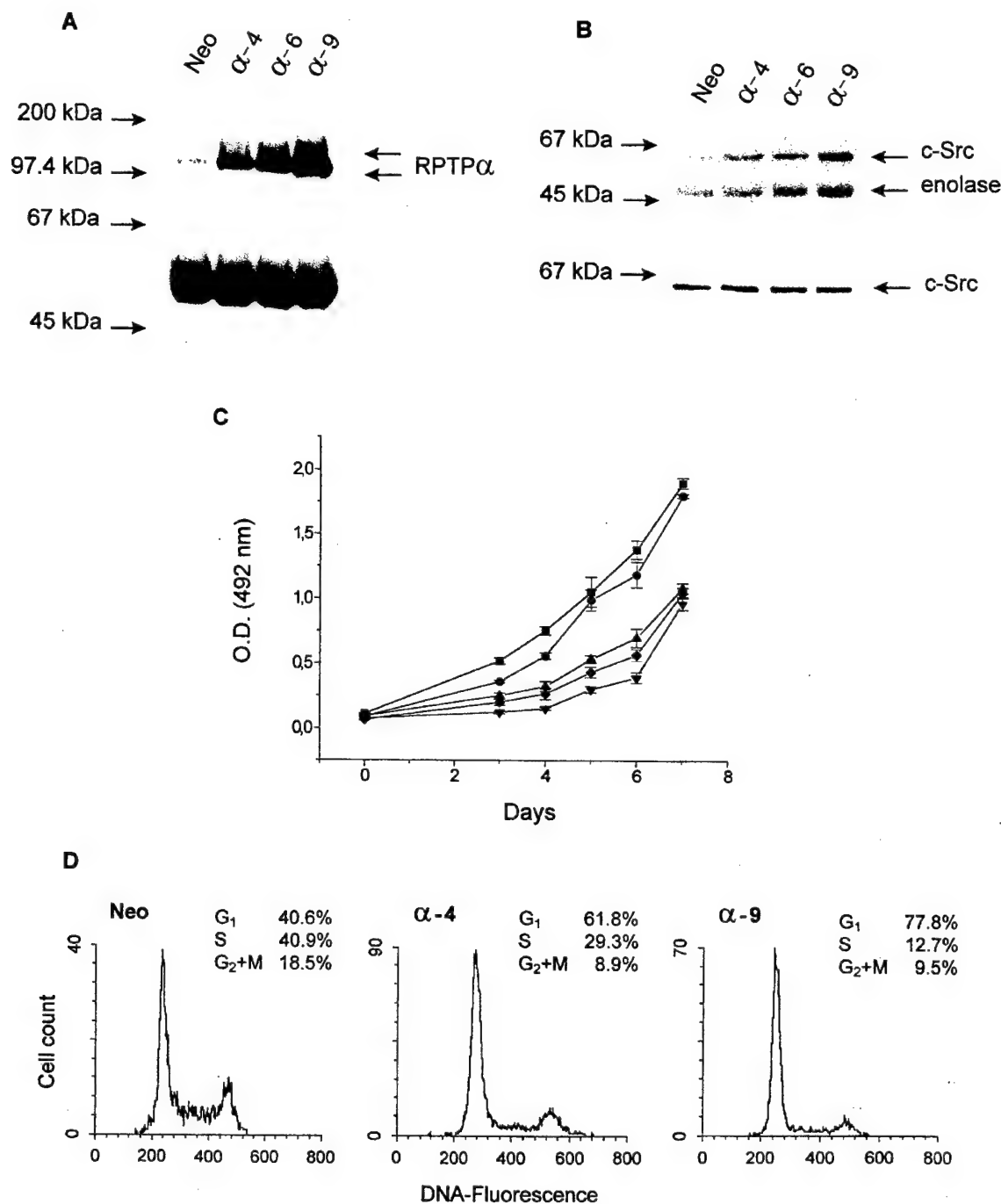
**Table 1** Pathological parameters of RPTP $\alpha$ -positive versus -negative human breast tumors

Parameter	RPTP $\alpha$ not overexpressed	RPTP $\alpha$ overexpressed	
Number of cases	36	15	
Age > 55 years	44% (16/36)	47% (7/15)	
Tumor grade III	53% (18/34)	20% (3/15)	( $P=0.02$ )
grade II	47% (16/34)	73% (11/15)	
grade I	0% (0/34)	7% (1/15)	
Necrosis	50% (19/36)	47% (7/15)	
Proliferation index positive	32% (11/34)	15% (2/13)	( $P=0.15$ )
Size (< 2 cm)	14% (5/35)	33% (5/15)	( $P=0.09$ )
N+	81% (29/36)	67% (10/15)	( $P=0.15$ )
1-4	41% (12/29)	50% (5/10)	
5-8	28% (8/29)	40% (4/10)	
>8	31% (9/29)	10% (1/10)	( $P=0.15$ )
ER negative	37% (13/35)	20% (3/15)	( $P=0.04$ )
PgR negative	54% (19/35)	46% (7/15)	
HER2/neu positive	43% (9/21)	17% (2/12)	

The level of statistical significance was determined using Fisher's test. Not all parameters were available for all tumors, hence the instances were sometimes less than the total of 51 cases. N+: presence of pathological nodal metastasis (number of metastasis-containing lymph nodes). ER: Estrogen receptor. PgR: progesterone receptor

colonies counted following a 3 week selection. The number of clones arising from RPTP $\alpha$  transfection was lower than that obtained upon transfection with empty vector ( $66 \pm 9$  vs  $110 \pm 15$ ) (values averaged over two

experiments with three plates each). Subsequently, stable transfection was carried out, and individual clones isolated (Figure 3a). Three different RPTP $\alpha$  overexpressing clones were randomly chosen for



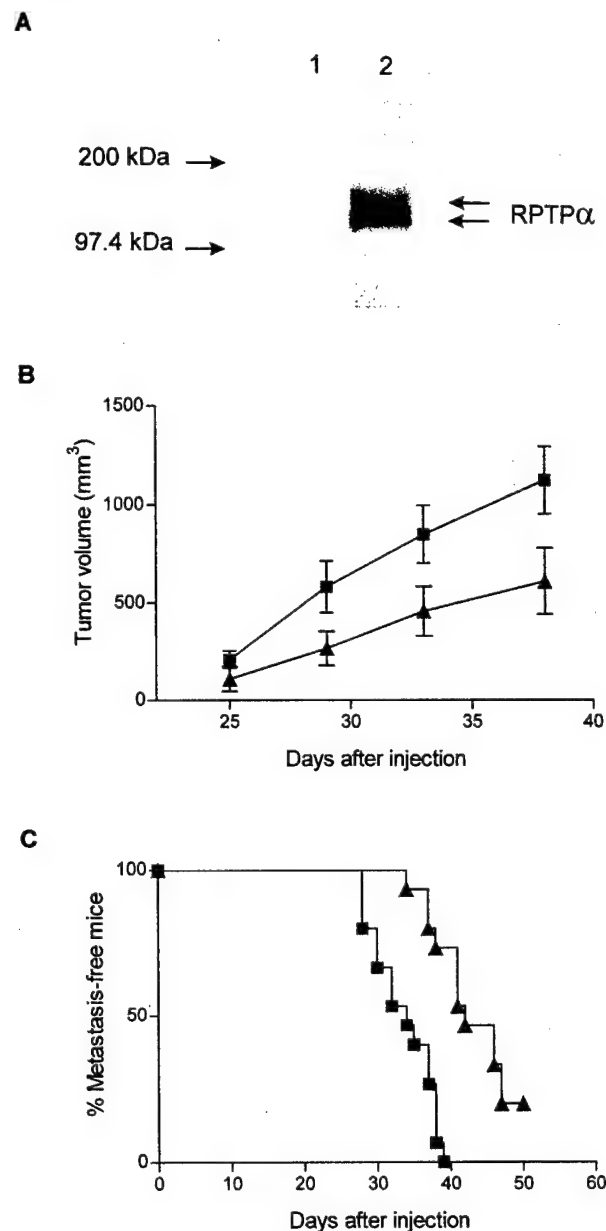
**Figure 3** RPTP $\alpha$  expression inhibits *in vitro* growth rate due to extension of G1 phase of the cycle. (a) Immunoblotting analysis of RPTP $\alpha$  expression in stably transfected MCF7 clones. Total protein lysates from an empty vector transfected clone (Neo) and from three RPTP $\alpha$  cDNA transfected clones ( $\alpha$ -4,  $\alpha$ -6, and  $\alpha$ -9) were immune precipitated with anti-RPTP $\alpha$  antiserum and blotted with the same antiserum. The smaller form of the protein corresponds to a differentially glycosylated precursor, which tends to become enriched in immune precipitations (Daum *et al.*, 1994). (b) Activation of c-Src kinase activity as a result of RPTP $\alpha$  expression. Top: *in vitro* kinase assay; c-Src was immune precipitated from an empty vector transfected cell clone (Neo) and 3 RPTP $\alpha$ -expressing clones ( $\alpha$ -4,  $\alpha$ -6,  $\alpha$ -9) and subjected to *in vitro* kinase reaction in the presence of  $\gamma$ -<sup>32</sup>P-labeled ATP, using enolase as exogenous substrate. Bottom: Anti-Src immunoblot; an aliquot of the c-Src immune precipitate was subjected to anti-Src immunoblotting. (c) Effect of RPTP $\alpha$  expression on growth rate. Parental MCF7 cells (■), a vector (control) transfected MCF7 cell clone (●), and three RPTP $\alpha$ -expressing clones (◆:  $\alpha$ -4; ▲:  $\alpha$ -6; ▼:  $\alpha$ -9) were seeded at equal initial densities, and growth measured using the Sulphorodamine B dye uptake method (Pizao *et al.*, 1992). The t=0 timepoint corresponds to 18 h after plating. A representative of three independent experiments is shown. Error bars indicate standard deviation (n=6 wells/time-point/clone). (d) Cell cycle distribution in an empty vector transfected clone (neo) and two RPTP $\alpha$  expressing lines ( $\alpha$ -4 and  $\alpha$ -9). Exponentially growing cells were trypsinized, fixed, and stained with propidium iodide, and fluorescence measured by flow cytometry

subsequent studies. Consistent with reports for other cell lines (den Hertog *et al.*, 1993; Zheng *et al.*, 1992), increased RPTP $\alpha$  expression led to elevated c-Src kinase activity (as measured by *in vitro* kinase assay *vis-a-vis* enolase as an exogenous substrate) (Figure 3b); this is presumably due to dephosphorylation by RPTP $\alpha$  of the negative regulatory site Y530 in c-Src.

Analysis of *in vitro* growth properties also revealed that the growth rate of RPTP $\alpha$ -expressing clones was significantly decreased in comparison with that of parental or empty vector-transfected cells (Figure 3c). The decrease of growth in the transfected clones, evident in the first days after plating, was not due to different plating efficiency as evaluated by number of adherent cells 18 h after plating (time-point 0 in Figure 3c). This observation was investigated further through cell cycle analysis by flow cytometry at 24 h from plating. Figure 3d shows a representative experiment, in which the results obtained for one control and two RPTP $\alpha$ -overexpressing clones ( $\alpha$ -4 and  $\alpha$ -9) are indicated. In three independent experiments, the proportion of cells in G<sub>0</sub>/G<sub>1</sub> was in the range of 35–40% for parental or empty transfected cells; by contrast, for RPTP $\alpha$ -overexpressing clones, the fraction of cells in G<sub>0</sub>/G<sub>1</sub> was increased to 62–78%. The proportion of cells in S phase was also reduced by RPTP $\alpha$ : for parental and empty transfected cells, values in the range of 40–44% were found, whereas in RPTP $\alpha$ -overexpressing clones the number in S phase was decreased to 29% (clone  $\alpha$ -4) and down to 13% in the highest expressing clone ( $\alpha$ -9). The absence of any sub-G<sub>1</sub> (hypodiploid) peak after propidium staining in our cell cycle analysis suggests that the growth inhibition is not accompanied by apoptotic cell death in RPTP $\alpha$ -overexpressing clones.

#### RPTP $\alpha$ expression reduces tumor growth and delays metastasis

The human MCF7 line is only poorly tumorigenic when inoculated into immuno-deficient test animals. Therefore, to analyse the consequences of RPTP $\alpha$  overexpression for tumor growth *in vivo*, we expressed RPTP $\alpha$  in N202.1A cells, a mouse line derived from a mammary tumor induced by transgenic expression of the HER2/neu protooncogene in the mammary epithelium (Lollini *et al.*, 1998). N202.1A cells were infected *in vitro* with a retrovirus encoding RPTP $\alpha$ , or (as the control) virus corresponding to the empty retroviral vector. Cells growing out after infection with either virus and the corresponding selection were pooled, so as to neutralize any effects of random clonal variability, and immunoblotting analysis confirmed the presence of RPTP $\alpha$  in cells infected with RPTP $\alpha$  encoding retrovirus (Figure 4a). Similar to the case for MCF-7 cells (Figure 3c,d), RPTP $\alpha$  expression in N202.1A cells resulted in significant inhibition of the *in vitro* proliferation rate (data not shown). Control or RPTP $\alpha$ -expressing N202.1A cell pools were then inoculated into 6-week-old Balb/c athymic mice, with the aim to monitor the effect of RPTP $\alpha$  expression on tumor growth (following subcutaneous injection), or on metastatic ability (following intravenous injection). As shown in Figure 4b tumor growth in animals injected subcutaneously with RPTP $\alpha$ -expressing cells was significantly reduced as compared to animals injected with



**Figure 4** Expression of RPTP $\alpha$  affects tumor growth *in vivo*. N202.1A cells (derived from a Neu-induced mammary tumor; Lollini *et al.*, 1998) were infected with empty control (■), or with an RPTP $\alpha$ -expressing retrovirus (▲). After selection, stably expressing pools of the respective cells were inoculated using the appropriate routes into nude mice. (a) Anti-RPTP $\alpha$  immunoblot (1: empty vector infected; 2: RPTP $\alpha$ -virus infected cells). (b) Tumor volume following subcutaneous injection. Mice (11/group) were inoculated in the right flank with  $5 \times 10^5$  cells/each, and tumors calibrated every 4 days between days 25 and 38. Error bars indicate standard deviations. A representative of two independent experiments is shown. (c) Development of lung metastasis following tail vein injection. Mice (15/group) were injected in the tail vein with  $10^6$  cells/each and examined daily for dyspnea, at which point the animal was sacrificed and lung metastasis confirmed by autopsy. The experiment was terminated at day 50. A representative of two independent experiments is shown; Chi-square analysis for this experiment indicated a *P*-value < 0.001 for the difference between the two survival curves

the control cell population. In a separate experiment, control or RPTP $\alpha$ -expressing cells were injected intravenously, and lung metastasis monitored by necropsy after animals developed dyspnea. All (15/15) mice injected with control cells developed lung



metastases within 39 days (median = 34); by contrast, the median delay until a similar metastasis burden for RPTP $\alpha$ -expressing cells was 43 days, with 3/15 animals still remaining symptom-free after 50 days (Figure 4c). Chi-square analysis revealed that this median delay of 9 days until metastatic symptoms was statistically significant at the  $P < 0.0001$  level.

## Discussion

Our observations show that a subclass (approximately one-third) of primary breast carcinomas is characterized by strongly elevated levels of RPTP $\alpha$ , and that this phenomenon is associated with lower tumor grade. To our knowledge, this is the first example of a study correlating expression level of a bona fide PTP with neoplastic disease status in humans. Furthermore, our experiments on breast cancer cells transfected with RPTP $\alpha$  show that enhanced RPTP $\alpha$  expression led to reduced growth rates *in vivo* and *in vitro*, an experimental effect not inconsistent with our clinical observation.

That enhanced RPTP $\alpha$  expression correlates with reduced tumor aggressiveness, and inhibits the growth rate and tumorigenicity of transfected cells was somewhat unexpected. The ability of RPTP $\alpha$  to activate the c-Src kinase (den Hertog *et al.*, 1993; Su *et al.*, 1999), sometimes with transforming effects (Zheng *et al.*, 1992, 2000), rather suggested a role for RPTP $\alpha$  as a potential oncogene itself, or as an enhancer of tumorigenicity. The recent finding of a Src-activating mutation in advanced human colon tumors (Irby *et al.*, 1999) indeed points at the existence of selective pressure towards c-Src activation in advanced tumors. Increased RPTP $\alpha$  mRNA expression was also noted in 10 out of 14 advanced (Dukes' stage D) colon adenocarcinoma samples (Tabiti *et al.*, 1995); however, this study did not include a comparison between more and less advanced tumors, and thus did not address the issue discussed here. A survey of various transgenic mouse models for mammary tumor development showed consistent overexpression of RPTP $\alpha$ , a PTP highly related to RPTP $\alpha$ , in HER2/Neu- or v-Ha-Ras-induced tumors (Elson and Leder, 1995b). These observations led to suggestions that increased PTP expression could constitute a tumor progression step, for instance by driving enhanced c-Src activity. However to what extent RPTP $\alpha$ , among the many potential regulators of c-Src, contributes to src activation in human tumor settings remains unestablished. In a pilot analysis of c-Src kinase activity in a subset of tumors analysed, we failed to observe a simple 1:1 correlation between RPTP $\alpha$  overexpression and c-Src activation (data not shown). A recent biochemical approach concluded that RPTP $\alpha$  is not the major Src-activating PTP in breast cancer cell lines (Egan *et al.*, 1999).

Several considerations may reconcile the apparent conflict between the Src-activating and fibroblast transforming capacity of RPTP $\alpha$ , and our present observations that RPTP $\alpha$  expression correlates with lower grade and reduces experimental tumor cell growth. First, RPTP $\alpha$ -induced Src activation may exert different effects in different settings or cellular contexts. Analogously, HER2/neu is tumorigenic in 3T3 fibro-

blasts (Di Fiore *et al.*, 1987), yet when overexpressed in MCF7 breast carcinoma cells leads to growth inhibition and differentiation (Giani *et al.*, 1998). One mechanism through which Src family kinases have been linked to negative growth regulation is phosphorylation of the Cbl protein, leading to degradation of PDGFR- $\alpha$  (Rosenkranz *et al.*, 2000). Second, whereas good evidence indicates that RPTP $\alpha$  activates c-Src, other thus far unidentified targets for RPTP $\alpha$  likely exist, whose dephosphorylation leads to growth arrest. Indirect evidence implicates RPTP $\alpha$  in inhibition of insulin signaling, through unknown mechanisms. Third, all human tumors analysed here had reached the stage of clinically recognizable disease. Therefore, it is possible that high RPTP $\alpha$  expression in a subset of tumors is a remnant of an earlier disease stage, where it may have contributed to initiation, or early progression, but is lost at later stages in favor of more aggressive progression events. Interestingly, transgenic expression of PTP $\alpha$  enhances the incidence of mammary malignancy, but the ensuing tumors express very low PTP $\alpha$  levels (Elson, 1999), consistent with a specific role of this PTP in early stages of progression only. Ultimately, transgenic models may be useful to further dissect the potential contribution of RPTP $\alpha$  to tumor initiation and/or progression.

The above considerations try to reconcile the transforming and c-Src activating potential of RPTP $\alpha$  with our observation that RPTP $\alpha$  expression preferentially occurs in low-grade tumors and inhibits tumor cell growth. Yet, more indirect connections are equally plausible. For instance, tumors that express RPTP $\alpha$  may belong to a biologically distinct subclass, for which RPTP $\alpha$  expression merely constitutes a marker. However, given the striking and unexpected negative effects of RPTP $\alpha$  expression on the growth of experimental tumors and on cell cycle distribution, it is tempting to propose that high RPTP $\alpha$  expression is the result of a feedback response to an alteration in cellular homeostasis. Under normal conditions, tyrosine phosphorylation is tightly regulated by the equilibrium between cellular PTKs and PTPs. A perturbation of this balance, for instance as a result of a specific oncogene activation, may lead to feedback responses including the increased expression of particular PTPs. Dual-specificity phosphatases that inactivate ERK kinases are induced as a consequence of the same stimuli that lead to ERK activation (Brondello *et al.*, 1997; Sun *et al.*, 1993). Increased expression of the PTPs LAR and PTP1B was observed in human breast epithelial cell lines as a consequence of HER2/neu expression (Zhai *et al.*, 1993), and LAR was shown to suppress transformation by this oncogene (Zhai *et al.*, 1995). RPTPs are also induced in many cases as a response to increased cell density, raising the possibility that this induction participates in contact inhibition (Ostman *et al.*, 1994). Hence, increased RPTP $\alpha$  expression could constitute a feedback response to an as yet unidentified tumorigenic insult, or stimulus. One tyrosine phosphorylation-mediated pathway activated in human breast tumors involves the HER2/neu oncogene (Tzahar and Yarden, 1998). Experimental murine HER2/neu-induced mammary tumors express high levels of the RPTP $\alpha$ -like PTP $\alpha$  (Elson and Leder, 1995b), and our study detected a weak and inverse correlation between RPTP $\alpha$  expression in human

tumors and HER2/neu positivity (Table 1). At any rate, we believe that the data presented in this paper warrant an exploration of the concept that PTPs in tumors act as non-constitutive, inducible tumor suppressors, whose expression is then lost again at further stages of progression.

The growth inhibition seen as a result of RPTP $\alpha$  expression is related to cell cycle arrest in G<sub>1</sub>. This suggests that a phosphoprotein involved in cell cycle control could be a specific substrate for RPTP $\alpha$ . v-Src is capable of inducing cyclin D1 in MCF7 cells (Lee *et al.*, 1999). It is unclear to what extent c-Src activation by RPTP $\alpha$  is comparable to v-Src expression, and the arrest-inducing effect of RPTP $\alpha$  may or may not be mediated through Src family kinases. A further molecular analysis of the cell cycle blocking effect of RPTP $\alpha$  will require more insight into the signaling pathways downstream of this widely expressed PTP. The membrane localization of RPTP $\alpha$  does not exclude the possibility that it could act on substrates localized in other compartments. RPTP $\beta$ , related to RPTP $\alpha$ , exists in both cytoplasmic and trans-membrane forms (Elson and Leder, 1995a). Furthermore, cleavage of the ectodomain of membrane PTPs can induce cellular redistribution of the catalytic domain (Aicher *et al.*, 1997).

An intriguing possible clue to the mechanism behind the ability of RPTP $\alpha$  to block cell cycle progression *in vitro* and experimental tumor growth rate *in vivo* is provided by the ability of RPTP $\alpha$  to block insulin responsiveness; this effect has now been observed in several cell types using various insulin response parameters: anti-adhesive effects in BHK cells (Moller *et al.*, 1995), prolactin promoter activation in GH4 pituitary cells (Jacob *et al.*, 1998), and GLUT4 translocation in adipocytes (Cong *et al.*, 1999). However, the target of RPTP $\alpha$  in insulin signaling is as yet unknown. Insulin-like growth factors are clearly implicated in progression through the cell cycle, proliferation, and inhibition of apoptosis of breast cancer cells *in vitro* (Ellis *et al.*, 1998; Werner and Le Roith, 1997). Moreover, fibroblasts lacking the IGF-1 receptor are resistant to transformation by dominant acting oncogenes (Sell *et al.*, 1994). The ensuing hypothesis, that the inverse correlation between RPTP $\alpha$  expression and mammary tumor progression reflects the potential of RPTP $\alpha$  to counteract the effects of insulin-like growth factors, is readily testable.

## Materials and methods

### Human tumor samples

Fifty-one samples of pathologically confirmed primary breast carcinoma, or neighboring non-diseased tissue from the same patient, were used. All patients were enrolled at the National Cancer Institute of Milan, and underwent surgery with complete axillary dissection. Mean tumor size at pathological examination was 33.7 mm (range 15–80). According to TNM classification (UICC, 1997), ten patients were classified for tumor size as pT1 ( $\leq 2$  cm) (20%), 34 as pT2 (2–5 cm) (68%), and six as pT3 ( $> 5$  cm) (12%). Twelve patients (23.5%) had no axillary lymph node involvement at histologic examination; among those with pathologic nodal metastasis, 17 (43.6%) had limited axillary metastatic involvement (1–4 metastatic nodes), whereas 22 (56.4%)

had a high number of metastatic nodes. All patients had infiltrating carcinoma: 40 (78%) had infiltrating ductal carcinoma, seven patients (14%) infiltrating lobular carcinoma, and the remaining four (8%) a less frequent histotype.

Tissues (tumor, or neighboring non-diseased tissue from the same patient) were collected within 5 min of surgical resection, snap frozen in liquid N<sub>2</sub> and stored at  $-80^{\circ}\text{C}$  until use. Primary tumor diameter and axillary nodal status were obtained from histopathological reports. Hematoxylin/eosin-stained histologic slides were reviewed for diagnostic reassessment of histotype, grading, necrosis, and proliferation index. Histologic grading was performed according to Elston, considering tubule formation, nuclear morphology and number of mitoses (Elston, 1984; Pereira *et al.*, 1995). The latter was scored as '+' in case of  $> 1$ , or '-' for  $\leq 1$ , mitosis per field after analysis of at least 10 microscopic fields at  $400\times$  magnification. Proliferation index was evaluated by [<sup>3</sup>H]-thymidine incorporation, with 2.3% of labeled cells being the cut-off value (Silvestrini *et al.*, 1985). Estrogen and progesterone receptor analysis relied on antibodies ER1D5 and 1A6 (both DBA, Italy), and scored according to (McCarty *et al.*, 1985). HER2/neu staining used antibody CB11 (YLEM, Italy), and scored positive when showing strong membrane labeling in  $> 20\%$  of tumor cells (cytoplasmic reactivity was not considered).

### Immunoblotting analysis

Specimens were homogenized and lysed for 1 h on ice in RIPA (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 10  $\mu\text{g}/\text{ml}$  leupeptin, 10  $\mu\text{g}/\text{ml}$  aprotinin). Lysates were clarified for 15 min at 15 000 g. Protein concentration was evaluated using the Bradford assay, and 100  $\mu\text{g}$  protein extract loaded for Western analysis. For immunoprecipitation, ProteinA-Sepharose was incubated with antibody for 1 h at  $4^{\circ}\text{C}$ , washed three times with RIPA, and then incubated with 1 mg of lysate for 2 h at  $4^{\circ}\text{C}$ . Immune complexes were washed three times with RIPA and eluted at  $95^{\circ}\text{C}$  in Laemmli buffer. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C, Amersham). Immunoblotting followed standard procedure, with visualization using an ECL system (Amersham). Anti-RPTP $\alpha$  antibodies used were: 443, against the intracellular domain; 35, against the C-terminus (residues 785–802); and 210, against residues 518–531 (numbering according to Kaplan *et al.* (1990)).

### Southern analysis

Genomic DNA was extracted using the SDS-Proteinase K method, digested with EcoRI, run on 0.8% agarose gel, and transferred to nitrocellulose. The probe (nucleotides 778–1107; Kaplan *et al.*, 1990) was prepared by EcoNI digestion of the human RPTP $\alpha$  cDNA, and labeled with  $\alpha$ -[<sup>32</sup>P]dCTP by random priming (Boehringer). Normalization for loading was achieved by re-hybridization with a  $\beta$ -actin probe and densitometric scanning to determine RPTP $\alpha$ / $\beta$ -actin signal ratios.

### Northern analysis

Total RNA was extracted (RNAzol B kit; Biotecx Laboratories), and 20  $\mu\text{g}/\text{sample}$  electrophoresed on a 1% agarose formaldehyde gel, transferred to nitrocellulose and immobilized by UV-cross-linking. Hybridization was performed in FBI-buffer (7% SDS, 1.5% SSPE, 10% PEG-8000) using as probe the entire human radiolabeled RPTP $\alpha$  cDNA. The membrane was washed with  $0.1\times\text{SSC}+0.1\%$  SDS for 20 min at  $25^{\circ}\text{C}$  followed by 20 min at  $42^{\circ}\text{C}$ . After stripping, the membrane was re-hybridized with a  $\beta$ -actin probe, and RPTP $\alpha$ / $\beta$ -actin ratios determined by densitometric scanning.

# Cells and transfections

Human breast carcinoma MCF7 and human kidney 293T cells were obtained from ATCC (Rockville, MD, USA). The mouse mammary carcinoma cell line N202.1A (derived from HER2/neu protooncogene transgenic mice) was provided by Dr PL Lollini (Lollini *et al.*, 1998). All cells were maintained in DMEM + 10% FCS. Full length mouse RPTP $\alpha$  cDNA was excised with *Xho*I and *Tha*I, and subcloned between the *Xho*I and *Eco*RV sites of pcDNA3/neo (Invitrogen). MCF7 cells were transfected with pcDNA3/RPTP $\alpha$ (wt) or with pcDNA3/neo (control) using calcium phosphate, and single colonies expanded in G418 (500  $\mu$ g/ml). For colony assays, MCF7 cells ( $5 \times 10^5$  cells/100 mm plate, seeded 24 h before transfection) were transfected in duplicate by lipofectin-mediated gene transfer (Life Technologies). After 3 weeks of selection in G-418, plates were stained using Diffquick (Baxter). N202.1A cells were infected with pLXSHD-derived, RPTP $\alpha$ -expressing or control retroviruses (Su *et al.*, 1996) and selected in 4 mM histidinol.

# Proliferation assay

Equal numbers of cells were seeded in 96-well plates, and the first time-point ( $t=0$ ) taken 18 h after seeding. Wells were fixed for 1 h at 4°C in ice cold 10% TCA. Cells were then washed with PBS, and incubated for 30 min with 0.4% Sulphorodamine B in 1% acetic acid (100  $\mu$ l/well). After three washes in 1% acetic acid, the dye was dissolved in 10 mM Tris pH 10.5 (100  $\mu$ l/well) and spectrophotometrically evaluated at 492 nm (Pizao *et al.*, 1992).

# Src kinase assay

Lysates were subjected to immune precipitation with anti-Src antibody 327 (Oncogene Science). Immune complexes were washed three times with RIPA, and once with kinase buffer (20 mM HEPES pH 7.0, 10 mM MnCl<sub>2</sub>). One half of each immunoprecipitate was incubated in 50  $\mu$ l kinase buffer containing 5  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP and 12.5  $\mu$ g acid-denatured enolase at 37°C for 5 min; the reaction was stopped by adding an equal volume of loading buffer and the proteins resolved by 10% SDS-PAGE followed by autoradiography. The other half of each immunoprecipitate was subjected to anti-c-Src immunoblotting.

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# Cell cycle analysis

Exponentially growing cells were trypsinized and collected in PBS. After 15 min of fixation in 50% methanol, cells were incubated at 37°C for 30 min. in the presence of 1 mg/ml ribonuclease A. DNA staining was performed at 0°C by 30 min incubation with propidium iodide (50  $\mu$ g/ml) in PBS containing 0.03% bovine serum albumin. Fluorescence was measured using a FACScan flow cytometer and data analysed using CellFIT Software (Becton-Dickinson).

# Tumorigenicity and experimental metastasis

Six-week-old Balb/c athymic mice were purchased from Charles River (Calco, Italy). Care and use of the animals was in accordance with institutional guidelines. Mice (11 animals/cell type) were injected subcutaneously in the right flank with  $5 \times 10^5$  cells each, and tumors calibrated at regular intervals. Tumor volume was calculated as  $0.5 \times d_1^2 \times d_2$  ( $d_1$  and  $d_2$  being the larger and smaller diameter, respectively). For experimental metastasis, mice were injected in the tail vein with  $1 \times 10^6$  cells each (15 animals/cell type), examined daily, and sacrificed when dyspnea was observed.

# Abbreviations

PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase; SFK, Src family kinase.

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# Receptor protein tyrosine phosphatase $\alpha$ activates Src-family kinases and controls integrin-mediated responses in fibroblasts

Jing Su\*, Madhavi Muranjan and Jan Sap

**Background:** Fyn and c-Src are two of the most widely expressed Src-family kinases. Both are strongly implicated in the control of cytoskeletal organization and in the generation of integrin-dependent signalling responses in fibroblasts. These proteins are representative of a large family of tyrosine kinases, the activity of which is tightly controlled by inhibitory phosphorylation of a carboxy-terminal tyrosine residue (Tyr527 in chicken c-Src); this phosphorylation induces the kinases to form an inactive conformation. Whereas the identity of such inhibitory Tyr527 kinases has been well established, no corresponding phosphatases have been identified that, under physiological conditions, function as positive regulators of c-Src and Fyn in fibroblasts.

**Results:** Receptor protein tyrosine phosphatase  $\alpha$  (RPTP $\alpha$ ) was inactivated by homologous recombination. Fibroblasts derived from these RPTP $\alpha^{-/-}$  mice had impaired tyrosine kinase activity of both c-Src and Fyn, and this was accompanied by a concomitant increase in c-Src Tyr527 phosphorylation. RPTP $\alpha^{-/-}$  fibroblasts also showed a reduction in the rate of spreading on fibronectin substrates, a trait that is a phenocopy of the effect of inactivation of the *c-src* gene. In response to adhesion on a fibronectin substrate, RPTP $\alpha^{-/-}$  fibroblasts also exhibited characteristic deficiencies in integrin-mediated signalling responses, such as decreased tyrosine phosphorylation of the c-Src substrates Fak and p130<sup>cas</sup>, and reduced activation of extracellular signal regulated (Erk) MAP kinases.

**Conclusions:** These observations demonstrate that RPTP $\alpha$  functions as a physiological upstream activator of Src-family kinases in fibroblasts and establish this tyrosine phosphatase as a newly identified regulator of integrin signalling.

## Background

Genetic inactivation of c-Src or Fyn, or transformation by v-Src — a gain-of-function mutation — affect cell adherence and cytoskeletal organization; these effects correlate with altered levels of tyrosine phosphorylation of proteins thought to mediate integrin signalling or control cytoskeletal architecture [1–3]. Src-family kinases clearly mediate integrin-dependent adhesive responses [4–8] because, during cell attachment on fibronectin matrices, c-Src is required for normal cell spreading [9], whereas Fyn is required for extracellular signal regulated kinase (Erk) activation [10].

A key mechanism controlling the activity of Src-family kinases is the reversible phosphorylation of a carboxy-terminal tyrosine residue — Tyr527 in chicken c-Src — that is characteristic of this enzyme family. This phosphorylation event, which is mediated by separate kinases (such as Csk and Chk [11,12]), favours the adoption of a conformational state associated with reduced catalytic activity, increased detergent solubility, and altered intracellular localization. Loss of this phosphorylation-based inhibitory

switch, for example, by deletion or mutation of the Tyr527 phosphorylation site, is a common mechanism that leads to oncogenic activation of c-Src [13]. Conversely, dephosphorylation of Tyr527 by a specific protein tyrosine phosphatase (PTPase) may be a mechanism favouring the adoption of the 'open' kinase-active state. Such a mechanism appears to account for the requirement for the PTPase CD45 in antigen-receptor signalling pathways involving Lck and Fyn in lymphocytes [14,15]. CD45 may function in a different manner with respect to other Src-family kinases in macrophages, however [16].

RPTP $\alpha$  is a widely expressed transmembrane receptor protein tyrosine phosphatase [17] that can dephosphorylate Tyr527 of c-Src *in vitro*, and that can lead to increased c-Src kinase activity and transformation when overexpressed [18,19]. It has also been reported that RPTP $\alpha$  overexpression can modulate cell adhesion [20,21]. By relying on drastic overexpression, however, such studies have failed to provide reliable insight into the role of endogenous RPTP $\alpha$  in these processes or in signalling pathways involving the normal function of Src-family

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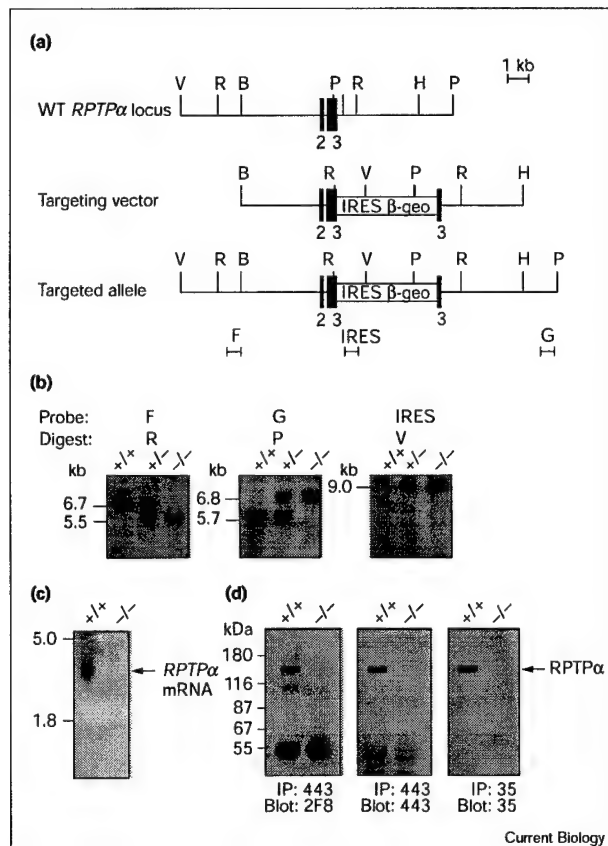
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Figure 1



Generation of an *RPTPα* null allele by gene targeting. **(a)** Schematic representation of the targeting vector and the structure of the wild-type (WT) *RPTPα* locus surrounding exons 2 and 3 [38] before (top) and after (bottom) homologous recombination. β-geo, β-galactosidase-neomycin-resistance fusion gene [39]; B, *Bam*HI; H, *Hind*III; P, *Pst*I; R, *Eco*RI; V, *Eco*RV; IRES, internal ribosomal entry site. The regions detected by probes F, G and IRES are also indicated. **(b)** Genotyping of wild-type (+/+), heterozygous *RPTPα*<sup>+/-</sup> (+/-), and homozygous *RPTPα*<sup>-/-</sup> (-/-) mice by Southern hybridization using probes F, G, or IRES. **(c)** Northern hybridization of RNA from embryonic fibroblasts derived from *RPTPα*<sup>+/-</sup> or *RPTPα*<sup>-/-</sup> embryos, using as probe a 0.7 kb *Xba*I fragment corresponding to the membrane-proximal PTPase domain of *RPTPα*. The 3 kb *RPTPα* mRNA is indicated. **(d)** Absence of RPTPα protein from *RPTPα*<sup>-/-</sup> mouse brain. Mouse brain lysates derived from *RPTPα*<sup>+/-</sup> or *RPTPα*<sup>-/-</sup> animals were subjected to immunoprecipitation followed by immunoblotting using various combinations of antibodies recognizing different domains in RPTPα. The domains recognized by each antibody are as follows: 443, the entire intracellular domain; 2F8, the ectodomain; and 35, the carboxyl terminus. The 130 kDa RPTPα protein is indicated.

kinases. A deficiency in the kinase Csk can be partly rescued by null mutations of c-Src, thus firmly establishing the epistatic relationship between c-Src and its upstream modulator Csk [1]. By contrast, no definite functional link has thus far been apparent between c-Src and individual members of the PTPase family. We reasoned

that the criteria for the identification of the phosphatase that acts as a physiological upstream activator of c-Src in a particular lineage or process would be twofold. First, deletion of the phosphatase should cause decreased c-Src catalytic activity. Second (and most importantly), loss of the PTPase should generate a phenotype that mimics, or at least has some characteristics in common with, that resulting from a null mutation of c-Src. We therefore used homologous recombination in mouse embryonic stem (ES) cells to generate a null allele at the *RPTPα* locus. The phenotype observed in *RPTPα*<sup>-/-</sup> fibroblasts derived from these mice included impaired c-Src and Fyn kinase activity and reduced cellular responses to adhesion on fibronectin, consistent with RPTPα acting as an upstream activator of Src-family kinases during this process.

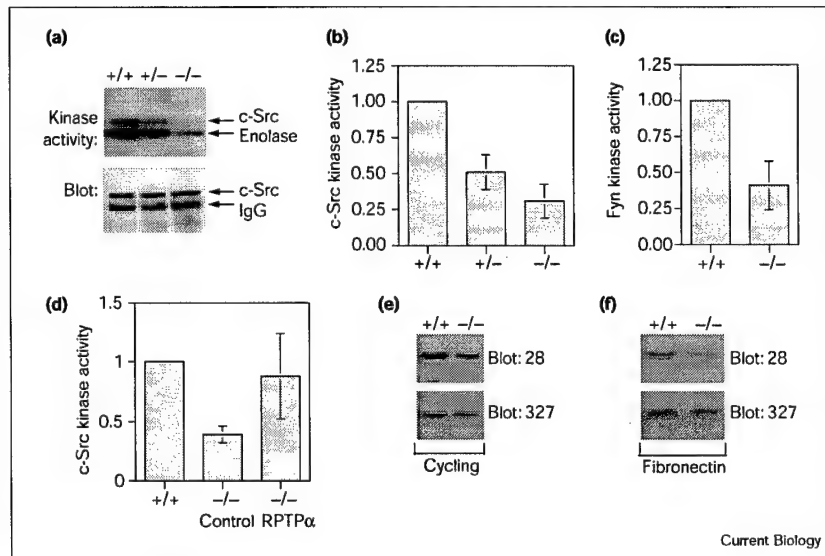
## Results

A β-galactosidase-neomycin-resistance (β-geo) fusion gene, preceded by an internal ribosomal entry site (IRES), was introduced into exon 3 (corresponding to the RPTPα extracellular domain) of the mouse *RPTPα* locus by homologous recombination (Figure 1a,b). Homozygous RPTPα mutant progeny were born in accordance with expected Mendelian ratios (data not shown). RNA analysis, using a probe corresponding to the region encoding the RPTPα membrane-proximal phosphatase domain, revealed no detectable levels of *RPTPα* mRNA in homozygous mutant embryonic fibroblasts (Figure 1c). Furthermore, protein analysis, using a battery of antibodies to various domains in the RPTPα protein, failed to detect any cross-reactive protein species in various tissues and cells from homozygous mutant animals, demonstrating the generation of a null allele (Figure 1d). This analysis also failed to detect evidence for the secretion of a small soluble RPTPα extracellular-domain fragment that might still be produced from the mutant locus upstream of the insertion site (data not shown). *RPTPα*<sup>-/-</sup> animals survived to adulthood and were fertile, but displayed gender-specific changes in body size, as well as nurturing defects. A full and detailed characterization of these whole-animal phenotypes will be reported at a later date.

To characterize the effects of *RPTPα* gene inactivation, we performed *in vitro* kinase activity measurements of c-Src and Fyn proteins immunoprecipitated from explanted, exponentially growing, primary fibroblasts derived from wild-type mice (+/+), *RPTPα*<sup>+/-</sup> heterozygous mice, or *RPTPα*<sup>-/-</sup> homozygous mice. These experiments revealed dose-dependent control of the activity of these kinases by RPTPα: the activities of c-Src and Fyn towards enolase—provided as an exogenous substrate—were reduced by 70% and 60%, respectively, in *RPTPα*<sup>-/-</sup> cells compared with wild-type cells (Figure 2a-c). This change in activity occurred in the absence of changes in c-Src or Fyn protein levels (Figure 2a). The c-Src kinase deficiency in cells derived from *RPTPα*<sup>-/-</sup> embryos was

Figure 2

RPTP $\alpha$  gene dosage affects c-Src and Fyn kinase activity in fibroblasts. The (a,b) c-Src or (c) Fyn proteins were immunoprecipitated from cells of the indicated genotypes at the RPTP $\alpha$  locus and their activity was assayed by measuring the amount of  $^{32}$ P incorporated into an exogenous substrate (enolase) *in vitro*. (a) Autoradiograph of a typical c-Src *in vitro* kinase assay, showing the incorporation into enolase, as well as autophosphorylation of c-Src (top panel), and a control anti-c-Src immunoblot of an aliquot of the immunoprecipitate (bottom panel) to show that equal amounts of protein were used in each assay. (b) Quantitation of c-Src kinase activity. Bars represent the average  $\pm$  standard error (SE) of three separate experiments. (c) Quantitation of Fyn kinase activity. Bars represent the average  $\pm$  SE of four separate experiments. Data in (a–c) were confirmed using several independently isolated primary or established fibroblast cultures. (d) Restoration of c-Src kinase activity by reintroduction of RPTP $\alpha$  into RPTP $\alpha$ <sup>−/−</sup> fibroblasts. RPTP $\alpha$ <sup>−/−</sup> cells were transfected with empty vector (control) or an RPTP $\alpha$  expression plasmid, and individual clones assayed for c-Src activity using the *in vitro* kinase reaction described in (a–c). Data are an average of three clones for cells transfected with control vector and two for the RPTP $\alpha$ -expressing clones. In (b–d) the c-Src or Fyn kinase activity in RPTP $\alpha$ <sup>+/+</sup> cells was taken as 1. (e,f) Difference in



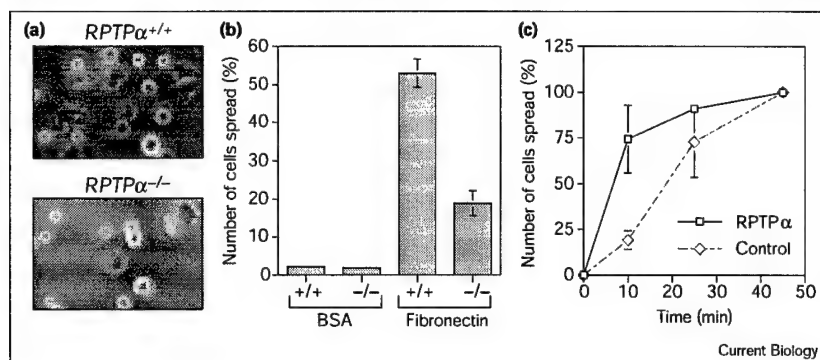
rescued by reintroduction of exogenous RPTP $\alpha$  (Figure 2d), and rescue of c-Src kinase activity by RPTP $\alpha$  was dependent on the dimerization state of the RPTP $\alpha$  protein (G. Jiang, J. den Hertog, J. Su, J. Noel, J. Sap, and T. Hunter, unpublished observations). Reduced c-Src kinase activity was also observed in tissue lysates from RPTP $\alpha$ <sup>−/−</sup> animals (data not shown).

To determine whether the effect of RPTP $\alpha$  ablation on c-Src kinase activity was associated with changes in phosphorylation of Tyr527 in c-Src, we made use of a monoclonal antibody, clone 28, that specifically recognizes the active (non-Tyr527-phosphorylated) form of c-Src. This antibody recognizes the motif surrounding Tyr527 in a manner that is hindered by phosphorylation of Tyr527 [22]. Equal amounts of lysate from exponentially growing wild-type or RPTP $\alpha$ <sup>−/−</sup> cells were subjected to immunoprecipitation with antibody 327, which recognizes an epitope in the Src homology 3 (SH3) domain of c-Src. The immunoprecipitate was subsequently halved and each half immunoblotted either with antibody 327 (as a control to normalize for the total amount of c-Src protein), or with clone 28. These experiments revealed a higher reactivity

of c-Src with the activation-specific antibody (clone 28) in wild-type cells compared with their RPTP $\alpha$ <sup>−/−</sup> counterparts (Figure 2e). Higher reactivity to clone 28 in wild-type cells compared with RPTP $\alpha$ <sup>−/−</sup> cells was also observed in total cell lysates during short-term adhesion assays on fibronectin (Figure 2f).

c-Src<sup>−/−</sup> fibroblasts have previously been shown to display a delay in adhesion-dependent spreading on fibronectin-coated substrates [9]. This effect can be rescued by kinase-negative versions of c-Src, demonstrating that physiological c-Src functions rely on adaptor-like mechanisms as well as on kinase activity. We explored whether c-Src and RPTP $\alpha$  might be linked in a common signalling cascade by determining whether the absence of RPTP $\alpha$  would mimic the fibroblast-spreading-deficiency phenotype characteristic of c-Src ablation [9]. Lack of RPTP $\alpha$  did not affect the efficiency of cell attachment to a fibronectin-coated surface (data not shown), suggesting that the integrin-adhesive function *per se* was not detectably affected. By contrast, following the attachment phase, RPTP $\alpha$ <sup>−/−</sup> cells displayed a clear but transient delay in cell spreading, which was most prominent between

Figure 3



RPTP $\alpha$  ablation delays the integrin-mediated cell spreading response of fibroblasts. (a) *RPTPα*<sup>+/+</sup> and *RPTPα*<sup>-/-</sup> cells 15 min after plating onto fibronectin-coated surfaces. (b) *RPTPα*<sup>+/+</sup> and *RPTPα*<sup>-/-</sup> cells were allowed to adhere for 15 min to bovine serum albumin (BSA)- or fibronectin-coated surfaces, and cell spreading expressed as a fraction of total adherent cells. (c) Accelerated *RPTPα*<sup>-/-</sup> cell spreading upon reintroduction of wild-type RPTP $\alpha$ . *RPTPα*<sup>-/-</sup> cells were infected with a retrovirus expressing RPTP $\alpha$  or with empty control vector and cell spreading was monitored over time after plating onto a fibronectin-coated surface.

10 and 20 minutes (Figure 3a,b), similar to that reported for *c-src* gene ablation [9]. In a rescue experiment, infection of *RPTPα*<sup>-/-</sup> cells with an RPTP $\alpha$ -expressing retrovirus (but not with a control virus) enhanced the spreading rate (Figure 3c), supporting the notion that the spreading deficit in *RPTPα*<sup>-/-</sup> cells is reversible, and a relatively direct result of the lack of RPTP $\alpha$ .

To determine whether deficiencies in proximal integrin-dependent signal transduction could account for the reduced spreading rate of *RPTPα*<sup>-/-</sup> fibroblasts, we monitored the levels of protein phosphorylation on tyrosine residues during the spreading process. Anti-phosphotyrosine immunoblotting analysis of total cellular lysates prepared at various stages of spreading revealed reduced tyrosine phosphorylation of proteins in the 130 kDa (Figure 4a, upper panel) and 40 kDa (Figure 4a, middle panel) range in cells lacking RPTP $\alpha$ , supporting the notion that RPTP $\alpha$  functions as an upstream activator of a tyrosine kinase during this process. Focal adhesion kinase (Fak), a protein tyrosine kinase, and the Crk-associated substrate p130<sup>cas</sup>, a multi-adaptor docking protein, were both identified as proteins the phosphorylation of which is markedly enhanced in v-Src-transformed cells; in non-transformed cells, both proteins are phosphorylated by, and associate with, c-Src in response to integrin-dependent cell adhesion, probably mediating some of the integrin-dependent events [2–4]. Immunoprecipitation with the respective specific antibodies revealed reduced tyrosine phosphorylation of p130<sup>cas</sup> and Fak in *RPTPα*<sup>-/-</sup> cells, consistent with impaired c-Src function (Figure 4b,c). Concomitant with tyrosine kinase activation, integrin-dependent fibroblast responses also include activation of the Erk arm of the mitogen-activated protein (MAP) kinase cascade [5,7], either through recruitment of the Grb2 adaptor to Fak–c-Src complexes [8,23], or through Fyn-mediated tyrosine phosphorylation of the adaptor protein Shc [10,24]. In accordance with this, attachment and spreading of wild-type fibroblasts on a fibronectin

substrate was accompanied by enhanced Erk activity, as assessed by phosphorylation of exogenously supplied myelin basic protein (MBP) in anti-Erk immunoprecipitates. By contrast, Erk activation was significantly reduced as a consequence of RPTP $\alpha$  ablation (Figure 4d,e).

## Discussion

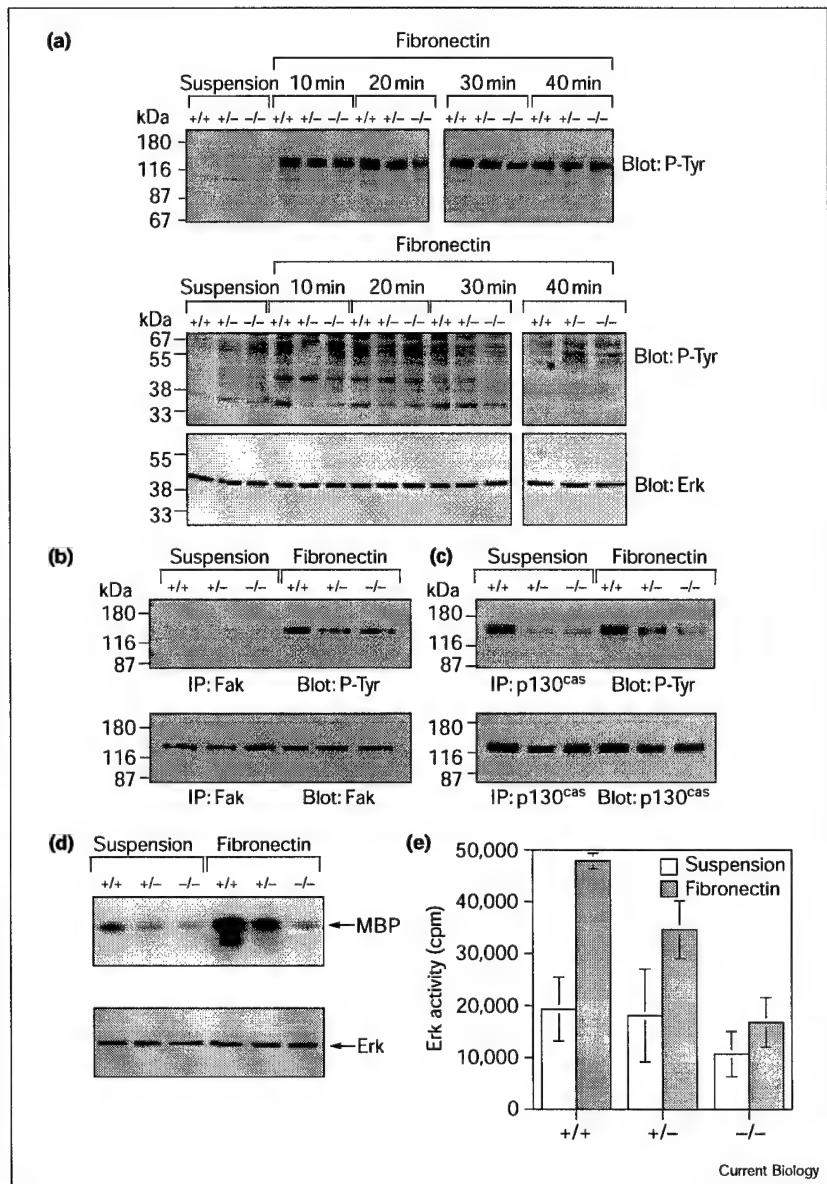
Although we cannot exclude additional mechanisms — such as the involvement of an intermediate phosphatase, a role for RPTP $\alpha$  in regulating Csk, or the ability of other PTPases to contribute to the control of c-Src tyrosine phosphorylation or activity [25–28] — the most economic interpretation of our observations is that RPTP $\alpha$  directly dephosphorylates c-Src Tyr527 *in vivo*, consistent with its ability to do so *in vitro* [18,19]. This dephosphorylation event would then either initiate, or maintain, the open conformation of c-Src. Such a direct relationship would also be consistent with the reported association of RPTP $\alpha$  with c-Src or Fyn [21,29]. We propose that our observations that RPTP $\alpha$  inactivation reduces c-Src and Fyn kinase activity, while also generating a phenocopy of c-Src ablation in fibroblasts, strongly suggest that this phosphatase acts as a physiological upstream regulator of Src-family kinases in this lineage. Our data clearly do not, however, rule out the possible existence of additional targets for RPTP $\alpha$ .

The control of c-Src function by RPTP $\alpha$  is reminiscent of the contribution of CD45 towards Lck or Fyn activity in lymphocytes [30], but contrasts with the apparent negative control of Src-family kinase function by CD45 in macrophages [16]. The cytosolic PTPase SHP-1 has been implicated in the activation of c-Src in platelets and thymocytes [25], although it may also inactivate the Src-family kinases Lck and Fyn [26]. The PTPase SHP-2 has been shown to associate with c-Src in fibroblasts and HT-29 colon carcinoma cells, and to be capable of dephosphorylating Tyr527 *in vitro* [27]. Intriguingly, *SHP-2*<sup>-/-</sup> fibroblasts display a phenotype very similar to that of



**Figure 4**

Effect of RPTP $\alpha$  deletion on phosphorylation events following adhesion on fibronectin. (a) Equal amounts of protein lysates derived from wild-type (+/+), RPTP $\alpha$ <sup>+/-</sup> (+/-) and RPTP $\alpha$ <sup>-/-</sup> (-/-) fibroblasts, prepared at various times after adhesion to a fibronectin substrate, as indicated, were analyzed by anti-phosphotyrosine (P-Tyr; top and middle panels) or anti-Erk (lower panel) immunoblotting. (b) Anti-Fak and (c) anti-p130<sup>cas</sup> immunoprecipitates from cells in suspension or cells allowed to adhere on fibronectin for 20 min were analyzed by immunoblotting with anti-phosphotyrosine antibodies (top panels) or, as control, with the respective p130<sup>cas</sup> or Fak antisera (bottom panels). (d) Anti-Erk immune complexes, prepared from cells in suspension or cells allowed to adhere on fibronectin for 15 min, were halved, with one half being subjected to an *in vitro* Erk kinase reaction using myelin basic protein (MBP) as substrate (top panel), and the other half to anti-Erk immunoblotting (lower panel). (e) The Erk activities from three independent experiments such as those presented in (d) were quantitated and are shown as the average  $\pm$  SE.



RPTP $\alpha$ <sup>-/-</sup> cells, that is, delayed spreading on fibronectin-coated substrates, and impaired activation of integrin-induced signalling events such as activation of Src-family kinases, phosphorylation of focal adhesion proteins, and Erk activation [28,31]. The involvement of both RPTP $\alpha$  and SHP-2 in the regulation of c-Src activity and in fibroblast spreading clearly calls for a more precise dissection of the specific roles of these two phosphatases in integrin signalling, and in the complex processes that constitute focal adhesion formation and turnover, and cytoskeletal remodelling. One range of possibilities may involve both PTPases independently (possibly directly) controlling Src

Tyr527 phosphorylation levels. Under such a scenario, both would be essential, as a result of, for instance, their abilities to act on different Src populations, in different intracellular locations, or at a different stage of the spreading process. Fibroblast adhesion is indeed known to involve a c-Src translocation event [9]. Alternatively, SHP-2 and RPTP $\alpha$  may act sequentially (but not necessarily in this order), with only one of these PTPases (or possibly even yet another PTPase) directly dephosphorylating Tyr527. In this case, regulatory interactions between RPTP $\alpha$  and SHP-2 must be postulated, and these might involve direct dephosphorylation of one

PTPase by the other, or more indirect mechanisms involving intermediate proteins. At any rate, the positive roles of RPTP $\alpha$  and SHP-2 contrast with the recently proposed negative role of the tumor suppressor PTEN in cell spreading and in signalling responses downstream of integrin engagement by extracellular matrix molecules [32,33]. Fibroblasts lacking the PTPase PTP-PEST were also recently shown to display enhanced spreading rates when plated on fibronectin, together with enhanced tyrosine phosphorylation of Fak and p130<sup>cas</sup> [34].

An additional consequence of c-Src ablation in mice is osteopetrosis due to a functional deficit in the osteoclast lineage [35,36]. X-ray analysis of RPTP $\alpha$ <sup>-/-</sup> mice up to 9 month old has thus far failed to detect signs of osteopetrosis (data not shown). This apparent paradox may suggest that, in osteoclasts, the role of RPTP $\alpha$  in c-Src function is performed by other PTPases. For instance, RPTP $\epsilon$ , the PTPase most similar to RPTP $\alpha$ , displays a much more restricted expression pattern than RPTP $\alpha$ , but is actually abundant in differentiated osteoclasts [37]. Similarly, in T lymphocytes, activation of Fyn has been suggested to be largely mediated by the PTPase CD45 [14]. By analogy, phosphorylation of Tyr527 in c-Src can be mediated by at least two kinases with different expression patterns, Csk and Chk [11,12]. Alternatively, given the large number of processes in which c-Src has been implicated [6,7], the requirement for RPTP $\alpha$  in c-Src function may be stimulus-specific, indicating that fibroblast spreading on fibronectin and bone resorption by osteoclasts involve different regulators of c-Src. Taken together, these considerations suggest a testable model, in which coupling between particular PTPases and Src-family kinases may convey a measure of cell- and stimulus-specificity to signalling pathways.

## Materials and methods

### Homologous recombination

A replacement-type targeting vector, consisting of an IRES- $\beta$ -geo cassette [38] (I. Chambers, Edinburgh) inserted at a PstI site present in exon 3 of the mouse RPTP $\alpha$  locus [39], was electroporated into the mouse ES cell line W4 (A. Joyner, NYU). Positive clones were identified by Southern hybridization, chimeric founders mated with 129SvJ animals, and the mutated allele maintained on this background.

### Immunoprecipitations and antibodies

Cells were lysed in RIPA buffer (50 mM HEPES pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml aprotinin. Equal amounts of protein from cleared extracts were subjected to standard immunoprecipitation or immunoblotting procedures. Antibodies used were: clone 327 against c-Src (Oncogene Science); anti-Erk 1 and 2 (Santa Cruz); F15020 (Transduction Laboratories) against Fak; antiserum B against p130<sup>cas</sup> (T. Parsons, University of Virginia); anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology); anti-RPTP $\alpha$  443 and 35, raised against the entire RPTP $\alpha$  intracellular domain or a carboxy-terminal peptide, respectively; antibody 2F8, against the RPTP $\alpha$  ectodomain, was a gift from M. Thomas (Washington University, St. Louis); clone 28 antibody was most generously donated by H. Kawakatsu (UCSF).

### Src and Fyn kinase assays

Immune complexes of c-Src or Fyn were washed three times with RIPA lysis buffer followed by two washes with kinase buffer (20 mM PIPES pH 7.0, 5 mM MnCl<sub>2</sub>), and reacted in a volume of 50  $\mu$ l kinase buffer containing 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and 12.5  $\mu$ g acid-denatured enolase at 30°C for 5 min. After electrophoresis, phosphorylation of enolase was quantified with a Phosphor-Imager (Molecular Dynamics).

### Erk assay

Triton-buffer lysates were subjected to immunoprecipitation with anti-Erk 1 and 2 antisera, and the immune complexes washed twice with lysis buffer and twice with kinase buffer (10 mM Tris pH 7.4, 10 mM MgCl<sub>2</sub>). An *in vitro* kinase reaction was performed in 50  $\mu$ l kinase buffer containing 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and 12.5  $\mu$ g MBP. After incubation for 30 min at 30°C, the reaction was stopped by boiling in SDS-PAGE sample buffer, and the reaction products separated by SDS-PAGE. The radioactive content of <sup>32</sup>P-labeled MBP was determined using liquid scintillation counting.

### Cell culture

Primary embryonic fibroblasts were isolated from E13-E15 day old embryos, with RPTP $\alpha$ <sup>+/+</sup> and RPTP $\alpha$ <sup>-/-</sup> embryos and cells always being derived in parallel from the same pregnancy. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. For spontaneous immortalization, cells were trypsinized and reseeded every 3 days at a density of 10<sup>6</sup>/100 mm dish until crisis had been overcome. Alternatively, a retroviral vector expressing polyoma large T antigen was used to immortalize the cultures. All experiments shown were performed on several independently isolated lines as well as on primary isolates. For reintroduction of RPTP $\alpha$ , an RPTP $\alpha$ <sup>-/-</sup> culture was infected as a pool with an RPTP $\alpha$ -expressing or control retrovirus. Quantitative infection was established by fluorescence of a green fluorescent protein (GFP) marker coexpressed from the same vector.

### Cell spreading

Cells were trypsinized briefly, and the digestion stopped using 0.5 mg/ml trypsin inhibitor (Gibco). They were then resuspended (125,000 cells/ml) in DMEM, and plated in 4 ml adhesion medium on 6 cm tissue-culture dishes coated with fibronectin (5  $\mu$ g/ml of human fibronectin (Gibco) overnight at 4°C). Plates were incubated at 37°C for the designated periods of time, chilled on ice for 15 min, and five microscope fields were photographed and counted.

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## COMMENTARY

# Emerging issues in receptor protein tyrosine phosphatase function: lifting fog or simply shifting?

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## SUMMARY

Transmembrane (receptor) tyrosine phosphatases are intimately involved in responses to cell-cell and cell-matrix contact. Several important issues regarding the targets and regulation of this protein family are now emerging. For example, these phosphatases exhibit complex interactions with signaling pathways involving SRC family kinases, which result from their ability to control phosphorylation of both activating and inhibitory sites in these kinases and possibly also their substrates. Similarly, integrin signaling

illustrates how phosphorylation of a single protein, or the activity of a pathway, can be controlled by multiple tyrosine phosphatases, attesting to the intricate integration of these enzymes in cellular regulation. Lastly, we are starting to appreciate the roles of intracellular topology, tyrosine phosphorylation and oligomerization among the many mechanisms regulating tyrosine phosphatase activity.

Key words: Tyrosine phosphorylation, Phosphatase, Integrin, SRC

## INTRODUCTION

Phosphorylation of proteins on tyrosine acts as a reversible and specific 'switching' mechanism, forming or disrupting regulatory connections between signaling proteins (Pawson and Scott, 1997). Even brief inhibition of tyrosine dephosphorylation dramatically increases overall phosphotyrosine levels, causing a breakdown of normal control mechanisms. Hence, continuous protein tyrosine phosphatase (PTP) activity is essential for appropriate specificity and intensity of cellular signaling (Fischer, 1999).

Whereas protein tyrosine kinases (PTKs) were often identified on functional grounds, molecular identification of PTPs, perhaps tellingly, has had to rely on tenacious biochemical purification and low-stringency hybridization. We now know that tyrosine dephosphorylation is mediated by several enzyme classes: the PTP family proper (whose only known substrate is phosphotyrosine), the dual specificity phosphatases (which also act on phosphoserine, phosphothreonine and, in the case of PTEN, phospholipids), and the low-molecular-mass and Cdc25-like enzymes (Denu and Dixon, 1998). In an intriguing symmetry to receptor tyrosine kinases, many PTPs display a transmembrane topology having ectodomains that often exhibit similarities to cell adhesion molecules. The intracellular moiety of most of these 'receptor' PTPs (RPTPs) contains two tandem PTP-homology domains, of which the membrane-proximal (D1) domain accounts for the bulk of catalytic activity. The membrane-distal (D2) domain appears capable of modulatory

effects, but its contribution remains enigmatic, since catalytically crucial residues are not invariably present (Hooft van Huijsduijnen, 1998; Nam et al., 1999).

Many excellent reviews deal with the structure and reaction mechanisms of PTPs (Barford et al., 1998; Denu and Dixon, 1998), phylogeny (Hooft van Huijsduijnen, 1998; Ono et al., 1999; Shi et al., 1998), their genetics (Van Vactor et al., 1998), and their roles in control of mitogenicity (Chernoff, 1999), adhesion and migration (Angers-Loustau et al., 1999b), insulin signaling (Goldstein et al., 1998), development (den Hertog, 1999), lymphocyte signaling (Mustelin et al., 1999; Neel, 1997) and the nervous system (Arregui et al., 2000; Desai et al., 1998; Stoker and Dutta, 1998). Here, we highlight recent studies that are improving our understanding of the role of RPTPs in integrin signaling and adhesion-related events, and of the mechanisms controlling RPTP function.

## CD45 AND SRC FAMILY KINASES

### The simple view

Before elaborating on this topic, we should recapitulate how tyrosine phosphorylation regulates the activity of SRC family kinases (SFKs; Brown and Cooper, 1996; Hubbard, 1999). The two best-characterized tyrosine phosphorylation sites in SFKs perform opposing regulatory functions. They are found within the enzyme's activation loop (Y416 in chicken SRC; Y394 in LCK) and in the vicinity of the C terminus (Y527 and Y505 in SRC and LCK, respectively). The former site undergoes

autophosphorylation, which is crucial for achieving full kinase activity. By contrast, phosphorylation of the C-terminal site can be mediated by a different kinase (Csk) and inhibits SFK activity. Inhibition occurs through intramolecular interactions between phosphorylated Y527 and the SH2 domain in SRC (and between the SH3 domain and the SH2-kinase linker), which stabilize a non-catalytic conformation. SFKs can therefore be controlled both by conformational mechanisms – displacement of SH2/SH3-mediated interactions – and by changes in phosphorylation – dephosphorylation of pY416 impairs activity, whereas pY527 dephosphorylation promotes it (Fig. 1A).

CD45 is an RPTP whose role in lymphocyte signaling had been extensively studied even prior to elucidation of its catalytic function. Many studies, using CD45-deficient lines, have observed that its absence severely impairs T-cell activation by compromising many signaling events proximal to the T-cell receptor (TCR): tyrosine phosphorylation, inositol phosphate generation and  $\text{Ca}^{2+}$  mobilization. This requirement for CD45 for coupling of TCR engagement to downstream responses has focused attention on the SFK LCK (and to a lesser extent FYN) as a key CD45 substrate, given the crucial role of LCK in T-cell activation and its ability to be activated by dephosphorylation of its C-terminal regulatory site. In this model, CD45 dephosphorylates (presumably specifically) the inhibitory Y505 site in LCK, making available a pool of active LCK for recruitment to signaling complexes associated with TCR ligation (Trowbridge and Thomas, 1994).

How widely relevant this model is for other lineages or pathways remained unclear; in fact, it accounted poorly for several observations in lymphoid cells. First, documenting decreased LCK kinase activity following CD45 loss was problematic; rather, CD45-deficient lines often display *increased* LCK activity (Ashwell and D'Oro, 1999). Second, reduction of CD45 levels sometimes resulted in *increased* tyrosine phosphorylation levels (Volarevic et al., 1992). Third, cross-linking CD45 with components of the TCR complex can decrease T-cell activation (Ledbetter et al., 1988). Lastly, if its role is solely to provide, in a catalytic manner, a basal level of SFK activation, why is CD45 expressed so highly?

#### CD45 in lymphocytes and macrophages: revealing paradoxes?

The relationship between CD45 and LCK has now been extensively revisited in CD45-null animals, which has enabled analysis of primary cells rather than transformed lines, phenotypic comparison with other genotypes, and studies in other lineages. CD45-deficient strains manifest severely reduced peripheral T-cell counts. Importantly, thymocytes and peripheral T-cells that do accumulate signal poorly through the TCR (Byth et al., 1996; Kishihara et al., 1993; Stone et al., 1997; reviewed by Neel, 1997). Together with the increased thresholds for thymic selection (Mee et al., 1999), the *CD45*<sup>-/-</sup> phenotype is in many respects consistent with CD45 acting as a positive regulator of TCR signaling. In fact, some aspects of T-cell ontogeny and signaling in CD45-null animals are similar to the LCK-null phenotype, and can be rescued by transgenic expression of constitutively active LCK (Pingel et al., 1999; Seavitt et al., 1999). The lack of autoreactive-B-cell deletion and impaired B-cell responsiveness in *CD45*<sup>-/-</sup> animals are consistent with CD45 acting as a positive transducer in this

lineage as well, and with it playing a key 'threshold-setting' role during development (Cyster et al., 1996), although some aspects can be interpreted in terms of a negative function (Neel, 1997).

Biochemical studies pose an intriguing contrast with the genetic and biological evidence for an LCK-activating role of CD45. LCK in CD45-deficient thymocytes, although hyperphosphorylated on tyrosine and existing to a greater extent in its 'inactive' conformation (Stone et al., 1997), actually displays *increased* kinase activity in vitro (D'Oro and Ashwell, 1999). This observation is consistent with previous reports of increased LCK activity and Y394 phosphorylation in CD45-negative lines (reviewed by Ashwell and D'Oro, 1999). Confirmation of this phenomenon in primary cells now makes the possibility that this is a transformation-related artifact unlikely. Similarly, Katagiri et al. (1999) observed enhanced activity of the SFK LYN and hyperphosphorylation of the site in its activation loop in CD45-negative immature B-cells. Although indirect effects of CD45 cannot be ruled out (e.g. via other proteins that engage the SH2/SH3 domain of LCK or LYN), these observations compel us to consider the possibility that CD45 can dephosphorylate the activating as well as the inhibitory sites in SFKs (Fig. 1B). Presumably, the effect of dephosphorylation of the site in the activation loop is dominant over the effect on the inhibitory site, leading to a net suppression by CD45 of total LCK activity.

The above studies were restricted to antigen receptor signaling in lymphoid lineages. But what is the relationship between CD45 and SFKs in other lineages or pathways? In adhering primary *CD45*<sup>-/-</sup> macrophages, the in vitro activity of two other SFKs, LYN and HCK, is also increased (Roach et al., 1997). Here again, further analysis pointed to CD45 dephosphorylating not only the inhibitory C-terminal phosphorylation site but also the positively acting site in the activation loop of these kinases. Strikingly, integrin- $\beta_2$ -mediated adhesion and spreading actually occurred more rapidly in *CD45*<sup>-/-</sup> than in wild-type cells, which suggests CD45 is a net *negative* regulator of integrin signaling (but it is unclear to what extent this is a consequence of increased LYN or HCK activity). Integrin- $\alpha_5$  (but not - $\alpha_4$ )-mediated T-cell adhesion is also negatively regulated by CD45 (Shenoi et al., 1999).

#### Necessary postulates?

The discrepancy between decreased responsiveness and increased LCK kinase activity of *CD45*<sup>-/-</sup> cells begs an explanation. One resolution would invoke other critical CD45 substrates (e.g. FYN or a mediator of phosphoinositide metabolism; Volarevic et al., 1992). Alternatively, in vitro activity might poorly reflect in vivo function – for example, if a small pool of strongly activated kinase remains in a location or conformation incompatible with recruitment to its site of action. CD45 could differentially regulate distinct pools of a target SFK, which Biffen et al. (1994) also have confirmed experimentally.

A single PTP that can have opposing effects clearly constitutes a somewhat dissonant situation. Hence, we may need to understand what (depending on the pathway or lineage) determines whether a PTP will act predominantly on one site or the other in its target SFK, or what can endow it with net attenuating versus potentiating capability. Such mechanisms might be topological, as posited by Thomas (Roach et al., 1997; Thomas, 1999; Thomas and Brown, 1999; Fig. 1D), or



Table 1. Summary of data implicating different PTPs in integrin-mediated events

PTP	Cell type	Effect	Suggested target(s) or substrates	Reference
CD45	Bone marrow derived macrophages (primary)	CD45 colocalizes with $\beta 2$ integrin to adhesion sites; ( <i>CD45</i> exon 6) <sup>-/-</sup> cells initially spread faster, but fail to sustain adhesion	HCK, LYN	Roach et al., 1997
	Jurkat T-cells	Absence of CD45 enhances adhesion to fibronectin via $\alpha 5 \beta 1$ (not $\alpha 4 \beta 1$ )	?	Shenoi et al., 1999
RPTP $\alpha$	BHK (overexpressing insulin receptor)	Overexpression antagonizes insulin-induced cell rounding	Insulin receptor, others?	Moller et al., 1995
	A431 epidermoid carcinoma	Overexpression increases cell-substratum adhesion; antagonizes EGF-induced cell rounding	SRC (FYN, YES?)	Harder et al., 1998
	Embryonic fibroblasts	Delayed spreading of <i>RPTP<math>\alpha</math></i> <sup>-/-</sup> cells on fibronectin; reduced phosphorylation of p130cas, FAK; reduced ERK activation	SRC, FYN	Su et al., 1999
	NIH3T3 fibroblasts	Catalytically inactive RPTP $\alpha$ localizes to focal adhesions in a manner that requires the Y798 tyrosine phosphorylation site in RPTP $\alpha$	?	Lammers et al., 2000
LAR	MCF-7 breast adenocarcinoma	Localizes to focal adhesions (regions undergoing disassembly)	?	Serra-Pagès et al., 1995
	HeLa epitheloid carcinoma	Binds laminin/nidogen; LAR cross-linking inhibits laminin-induced cytoskeletal reorganization	?	O'Grady et al., 1998
	U2OS osteogenic sarcoma	Localizes to focal adhesions; overexpression causes apoptosis	p130cas	Weng et al., 1999
SHP-2	Embryonic fibroblasts	Delayed spreading and reduced migration of ( <i>SHP-2</i> exon 3) <sup>-/-</sup> cells on fibronectin; increased number of focal adhesions	FAK	Yu et al., 1998
		( <i>SHP-2</i> exon 3) <sup>-/-</sup> cells manifest delayed spreading on fibronectin; reduced phosphorylation of p130cas, FAK, paxillin; no integrin-mediated ERK activation	SRC (via SHPS-17)	Oh et al., 1999
	MCF-7 breast adenocarcinoma	Catalytically inactive SHP-2 reduces integrin-dependent chemotaxis and enhances focal adhesion formation	FAK	Manes et al., 1999
	Rat-1 fibroblast	Expression of catalytically inactive SHP-2 reduces integrin-mediated ERK activation	?	Tsuda et al., 1998
	Embryonic fibroblasts	Fibronectin stimulates SHP-2 recruitment to $\beta$ -PDGF receptor, PDGF-dependent ERK activation, and mitogenesis. Mutation of SHP-2 docking site in $\beta$ -PDGF receptor abolishes fibronectin sensitivity	Ras-GAP docking site in $\beta$ -PDGF-receptor	DeMali et al., 1999
	Aortic endothelial cells	Mutation of SHP-2 binding site in $\beta$ -PDGF-receptor reduces chemotaxis towards PDGF	FAK	Qi et al., 1999
SHP-1	Bone marrow derived macrophages (primary)	SHP-1-deficient (me <sup>v</sup> ) cells manifest increased (long-term) $\alpha_m \beta_2$ -mediated adhesion and spreading	PI3-kinase mediated	Roach et al., 1998
PTP-PEST	Rat1 fibroblasts	Overexpressors attach and spread normally on fibronectin, but display reduced motility	p130cas	Garton and Tonks, 1999
	Embryonic fibroblasts	PTP-PEST <sup>-/-</sup> cells spread faster, have more focal adhesions on fibronectin, and display reduced motility	p130cas, paxillin FAK, PSTPIP	Angers-Loustau et al., 1999a
PTP1B	293 cells	No cellular effect described	paxillin	Shen et al., 2000
	L-cells	Wild-type associates with $\beta 1$ -integrin; colocalizes with vinculin; no effect on adhesion, spreading, focal contacts. Catalytically inactive PTP1B reduces adhesion, spreading, and number of focal adhesions on fibronectin	SRC, FAK, paxillin	Arregui et al., 1998
	3Y1 fibroblasts	Overexpression of wild-type antagonizes spreading, focal adhesion formation, migration, and integrin-mediated ERK activation	p130cas	Liu et al., 1998
PTEN	3T3 fibroblasts, U87MG glioblastoma	Overexpression of wild-type inhibits integrin-dependent spreading, migration, formation of focal adhesions, and integrin-induced ERK activation	FAK, Shc	Tamura et al., 1999
YopH ( <i>Yersinia</i> )	HeLa epitheloid carcinoma	Destabilizes focal adhesions	p130cas, FAK	Black and Bliska, 1997; Persson et al., 1997

Note that the same PTPs often also intervene in other signaling pathways – e.g. CD45 in TCR-signaling, SHP-2 in growth factor and cytokine signaling (Feng, 1999), and LAR and PTP1B in cadherin-dependent events (Balsamo et al., 1996; Kypta et al., 1996; Muller et al., 1999).

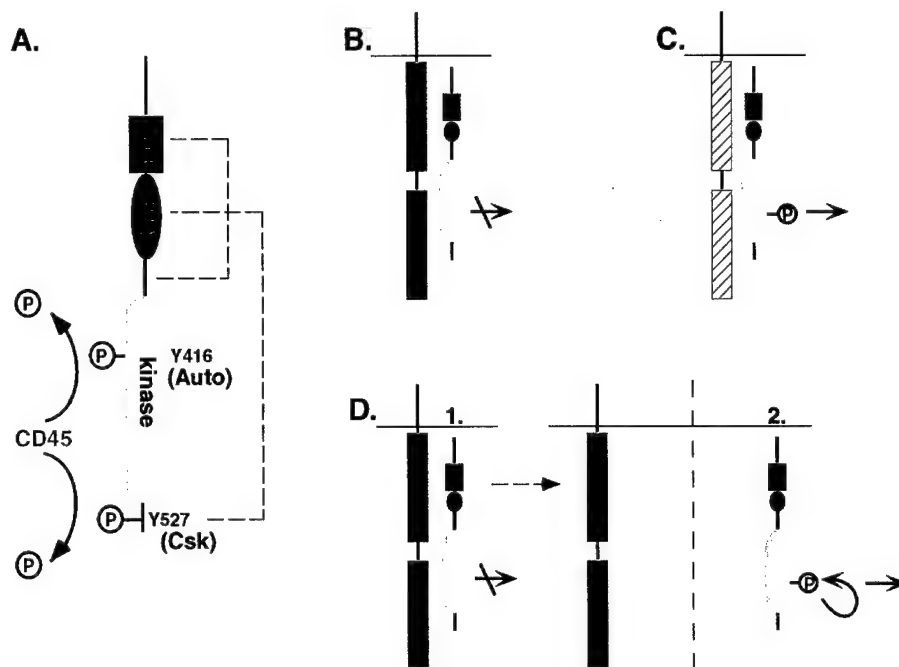
intrinsically catalytic (specifically directing activity towards one residue or the other; Fig. 1C).

With regard to a topological mechanism, it is useful to keep in mind that autophosphorylation of the pY416-type site can occur in trans. Therefore, doubly dephosphorylated LCK (kept inactive, and yet at the same time duly 'primed' by pY527 dephosphorylation) could undergo *regulated* activation through segregation and clustering of a subpopulation into a different, signaling, compartment. Here, 'sheltered' from continuing dephosphorylation of the site in the activation loop,

transphosphorylation of LCK and productive activation can occur (Fig. 1D). In this way, T-cell CD45 might act as an inhibitor of LCK outside of signaling complexes, but as a net functional activator within them. The observation that in macrophages, where it acts as a functional inhibitor of HCK and LYN during integrin signaling, CD45 is present *within* focal contacts, at which it colocalizes with  $\beta 2$ -integrin, is consistent with this hypothesis (Roach et al., 1997).

One way of excluding CD45 from signaling complexes might be assembly of lipid rafts (Brown and London, 1998). Recent

**Fig. 1.** Possible modes of interaction between CD45 and SRC family kinases (SFKs). (A) Domain structure of an SFK, such as LCK. The Y416-type site is an autophosphorylation site (Y394 in LCK) in the activation loop of the kinase domain; its phosphorylation can occur in trans and is necessary for effective kinase activity. By contrast, phosphorylation of the Y527-type site (Y505 in LCK) is mediated by another kinase and is inhibitory, since it favors intramolecular associations (dashed lines) that result in a kinase-inactive conformation. (B) Recent data suggest that both phosphorylation sites can be dephosphorylated by CD45. This might endow CD45 with the ability locally to inhibit SFK activity and SFK-mediated signaling. This effect might predominate where the SFK and the PTP are constitutively in each other's vicinity and/or where conformational mechanisms (SH2 and SH3 domain displacement) are more critical to SFK activation than dephosphorylation of the pY527-type site (e.g. the interaction between CD45 and HCK and LYN in macrophages; Roach et al., 1997). (C) CD45 might act as a net activator if mechanisms exist that can alter the CD45 protein so as to enhance its catalytic specificity towards the pY527-type site (as compared to the pY416-type site). For instance, tyrosine phosphorylation of the RPTP might perform such a function for RPTP $\alpha$  with respect to SRC (Zheng et al., 2000). (D) In a two-compartment model, CD45 might dephosphorylate both sites and act negatively in a non-signaling compartment (1). If the half-life of the dephosphorylated Y527-type site is sufficiently long, the SFK can segregate and cluster into a separate (signaling) compartment (2) from which CD45 is excluded. Here, autophosphorylation of the Y416-type site ensures local activation and signal transmission, again converting CD45 into a net activator.



observations indeed report that CD45 is absent from rafts containing LCK or aggregated TCRs (Janes et al., 1999; Rodgers and Rose, 1996). Alternatively, the mechanism segregating CD45 from LCK might be protein based. The CD45-associated protein CD45AP can affect the degree of association between CD45 and LCK (Matsuda et al., 1998). Exclusion might be a consequence of the architecture of supramolecular activation clusters (Monks et al., 1998). In this respect, parts of the CD45 ectodomain have mucin-like properties, which might exclude it from areas of close cell-cell apposition (Trowbridge and Thomas, 1994). Lastly, the ectodomain might perform an active role through cis or trans interactions with other membrane-associated proteins, thus allowing (or not) CD45 to be represented in particular plasma membrane subdomains (Kozieradzki et al., 1997; Leitenberg et al., 1996).

An intrinsic mechanism that might fine-tune the mode of interaction of RPTPs with SFKs is tyrosine phosphorylation of the RPTP itself. Observations in this respect on the potential significance of tyrosine phosphorylation of RPTP $\alpha$ , which can modulate both the intracellular localization and the intrinsic catalytic specificity of the enzyme, are discussed in more detail below.

## PTPS AND INTEGRIN SIGNALING IN FIBROBLASTS

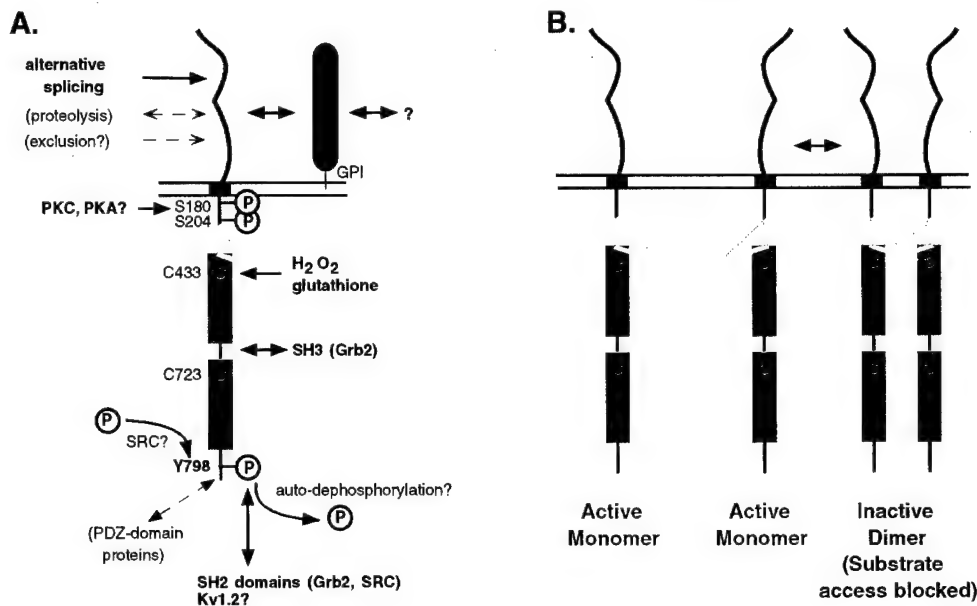
### RPTP $\alpha$ and SRC

SFKs perform a variety of functions in non-hemopoietic tissues

(Brown and Cooper, 1996; Lowell and Soriano, 1996). Is there evidence for their positive or negative control by RPTPs in these settings? Whereas the existence of few Y527 kinases would allow little opportunity for regulation by Y527 phosphorylation (Brown and Cooper, 1996), the size and complexity of the PTP family could provide numerous and flexible opportunities for regulation by dephosphorylation of this site.

Integrin signaling in fibroblasts involves crucial roles for SRC and FYN, and their regulated activation by PTPs has been postulated (Giancotti and Ruoslahti, 1999). Overexpression of RPTP $\alpha$  activates SRC (den Hertog et al., 1993; Zheng et al., 1992, 2000) and profoundly affects adhesive properties (Harder et al., 1998; Moller et al., 1995). Studies of embryonic fibroblasts from RPTP $\alpha$ <sup>-/-</sup> mice recently revealed a likely physiological role of RPTP $\alpha$  in SRC regulation and integrin signaling. Loss of RPTP $\alpha$  resulted in reductions in SRC and FYN kinase activities that were concomitant with increased phosphorylation of Y527 of SRC; reduced SRC activity was also observed in RPTP $\alpha$ <sup>-/-</sup> tissue extracts (Ponniah et al., 1999; Su et al., 1999). Given the ability of RPTP $\alpha$  to associate with SRC and FYN (Bhandari et al., 1998; Harder et al., 1998), direct dephosphorylation of the C-terminal inhibitory site might underlie the control of these kinases by RPTP $\alpha$ .

To what extent is the requirement for RPTP $\alpha$  in achieving normal SRC and FYN activities relevant? Full resolution of this issue will have to await more exhaustive analysis of the RPTP $\alpha$ <sup>-/-</sup> phenotype and its comparison with that of SFK



**Fig. 2.** Regulatory mechanisms that can affect RPTP activity. (A) The illustration centers on mechanisms documented for RPTP $\alpha$  (bold). Text in parentheses indicates mechanisms shown to be operational for other RPTPs (see text for illustrative references; C-terminal association with PDZ-domain containing proteins was reported by Kawachi et al., 1999). (B) Dimerization of RPTP $\alpha$  might inhibit activity because of mutual insertion of a structural wedge element (yellow) into the catalytic cleft that blocks substrate access (right). Note that no suggestions regarding the relationship between homodimerization, association with contactin, and other regulatory events are intended; whether changes in the dimer-monomer equilibrium affect phosphorylation or ectodomain-mediated associations (or vice versa) in fact remains to be investigated.

knockouts. Clearly, the fulminant osteopetrosis that is a major hallmark of SRC ablation is not observed in *RPTP $\alpha$ <sup>-/-</sup>* mice or in mice lacking RPTP $\epsilon$ , a close homologue of RPTP $\alpha$  highly expressed in osteoclasts (S. Pownall and F. Jirik, personal communication). Interestingly, PTP inhibitors show promise in treatment of osteoporosis (Schmidt et al., 1996). However, *RPTP $\alpha$ <sup>-/-</sup>* fibroblasts show a delay in spreading on fibronectin (Su et al., 1999) – a phenotype strikingly similar to that of *SRC<sup>-/-</sup>* fibroblasts (Kaplan et al., 1995). This *RPTP $\alpha$ <sup>-/-</sup>* defect is accompanied by impaired signaling responses downstream of integrin ligation (tyrosine phosphorylation of FAK and p130cas, and ERK activation are reduced). Hence, the biological and biochemical data support a net activating function for RPTP $\alpha$  with respect to SRC and FYN in fibroblast integrin signaling. Yet, overexpressed RPTP $\alpha$  is also capable of dephosphorylating pY416 in SRC (Zheng et al., 1992); therefore, RPTP $\alpha$  might also act in an inhibitory manner with respect to certain SFK-mediated processes.

### A multitude of PTPs

Recent years have witnessed an accumulation of reports that end up implicating a remarkably high number of PTPs in control of integrin signaling and/or SFK activity. The LAR protein resides at focal adhesions (Serra-Pagès et al., 1995; Weng et al., 1999), where it can interact with laminin-nidogen (O'Grady et al., 1998). The reduced SRC activity and impaired spreading on fibronectin characteristic of *RPTP $\alpha$ <sup>-/-</sup>* fibroblasts are strikingly similar to the effect of SHP-2 inactivation (Oh et al., 1999; Yu et al., 1998); this is consistent with other evidence implicating SHP-2 in SRC regulation (Walter et al., 1999) and integrin-dependent events (Table 1). Such cooperation between

a cytosolic and a transmembrane PTP in activating SRC introduces an intriguing level of complexity, and yet additional cytosolic PTPs might also be involved in SRC regulation (Moller et al., 1994; Somani et al., 1997). Similarly, lymphoid FYN and LCK appear subject to control by SHP-1 and PEP (besides CD45; Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999; Lorenz et al., 1996).

Several more cytosolic PTPs have now been shown to control integrin-mediated events in fibroblasts at other levels (Table 1). For instance, the SRC substrate p130cas can be dephosphorylated by PTP1B, PTP-PEST or LAR, and various candidate FAK phosphatases have also been proposed. This multiplicity of PTPs capable of affecting a particular substrate or pathway is also seen in other settings – for instance, cadherin-mediated signaling (Balsamo et al., 1996; Brady-Kalnay et al., 1995; Burden-Gulley and Brady-Kalnay, 1999; Fuchs et al., 1996; Kypta et al., 1996; Meng et al., 2000; Muller et al., 1999) and insulin responsiveness (Elchebly et al., 1999; Goldstein et al., 1998). If confirmed, this pattern might reflect functional overlap and redundancy, actions on different substrate subpopulations, or the existence of complex cascades of PTPs (given that many PTPs are themselves tyrosine phosphorylated) and kinases (e.g. Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999).

An additional conclusion is that both increased and reduced expression of a PTP can have similar consequences, as illustrated by the effect of PTP-PEST on cell motility (Angers-Loustau et al., 1999a; Garton and Tonks, 1999). Possibly, altering expression in either direction interferes with the appropriate rate of focal adhesion turnover required for optimal motility.



## DO RPTPS ALSO DEPHOSPHORYLATE SFK SUBSTRATES?

In spite of the bias of studies towards their action on SFKs, it is plausible that these are not the only RPTP targets. The  $\zeta$  chain of TCR is an additional candidate for an CD45 substrate (Furukawa et al., 1994; Kashio et al., 1998). Tissues from *RPTP $\alpha$ <sup>-/-</sup>* animals display hyperphosphorylation of several protein species (Ponniah et al., 1999; A. Petrone and J. Sap, unpublished). RPTP $\alpha$  associates with and dephosphorylates the K<sup>+</sup> channel Kv1.2 (Tsai et al., 1999), and is also a remarkably specific inhibitor of insulin receptor signaling (Jacob et al., 1998; Moller et al., 1995). An intriguing feature shared by the TCR  $\zeta$  chain and Kv1.2 is that both are themselves potential substrates for SFKs or for SRC-associated kinases, such as PYK2 (Felsch et al., 1998). This raises the possibility that RPTPs not only act on both activating and inhibitory sites within SFKs but perhaps also contribute to dephosphorylation of SFK substrates. Clearly, dephosphorylation of SFKs and their substrates by the same PTP will need to be well coordinated; again, they might be separated spatially (see the topological considerations discussed for CD45) or perhaps temporally.

## PTP REGULATION

The structural analogy with receptor tyrosine kinases naturally has sparked interest in the possibility that ligands modulate RPTP activity. A significant number of proteins are candidates for such ligands. Yet, before we elaborate on this issue, keep in mind that RPTPs already appear to be regulated by many other mechanisms, such as phosphorylation on serine/threonine (e.g. den Hertog et al., 1995; Ostergaard and Trowbridge, 1991; Tracy et al., 1995) and on tyrosine (e.g. Autero et al., 1994; den Hertog et al., 1994; Su et al., 1996; Toledano-Katchalski and Elson, 1999), proteolysis (e.g. Aicher et al., 1997), alternative splicing or 5'-exon usage (e.g. Daum et al., 1994; Elson and Leder, 1995; Kozieradzki et al., 1997), and reactive oxygen species (e.g. Barrett et al., 1999; Fialkow et al., 1997; Gross et al., 1999; Fig. 2A).

### Regulation by tyrosine phosphorylation

Work on RPTP $\alpha$  has now provided firm support for the relevance of tyrosine phosphorylation as a mechanism regulating RPTP activity. This RPTP is itself tyrosine phosphorylated on a C-terminal residue (Y798) and possibly others. Y798 phosphorylation creates a binding site on RPTP $\alpha$  for the adaptor protein Grb2 without concomitant Sos recruitment (den Hertog and Hunter, 1996; den Hertog et al., 1994; Su et al., 1994, 1996). A similar, but not identical, situation applies to PTPe (Toledano-Katchalski and Elson, 1999).

Importantly, mutation of Y798 in RPTP $\alpha$  into a non-phosphorylatable residue significantly alters the properties of this RPTP in PC12 (Su et al., 1996) and 3T3 cells (Lammers et al., 2000; Zheng et al., 2000), which suggests that tyrosine phosphorylation of this residue in RPTP $\alpha$  is of profound regulatory relevance. Phosphorylation of Y798 in RPTP $\alpha$  enhances its activity and specificity towards SRC pY527, as compared with pY416, through a phosphotyrosine-

displacement mechanism (Zheng et al., 2000). Moreover, it might mediate RPTP $\alpha$  localization to focal adhesions (although this could be shown only for catalytically inactive RPTP $\alpha$ ; Lammers et al., 2000) and the interaction of RPTP $\alpha$  with Kv1.2 (Tsai et al., 1999).

CD45 has also been reported to undergo tyrosine phosphorylation (Autero et al., 1994). Specific targeting towards particular phosphorylation sites in LCK, cellular locations or substrates by regulated tyrosine phosphorylation of CD45 might therefore also contribute to the resolution of some of the paradoxes regarding this RPTP discussed above.

### Ligand binding and dimerization

The numerous cis and trans interactions proposed to engage CD45 have been discussed (Trowbridge and Thomas, 1994). RPTP $\alpha$  associates in cis with contactin (Zeng et al., 1999), a GPI-linked adhesion molecule with which RPTP $\beta$ /PTP $\zeta$  also associates in trans (Peles et al., 1998). Other RPTP ectodomains associate homophilically with their counterparts on neighboring cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994), with neuronal CAMs (Peles et al., 1998; Sakurai et al., 1997) and with components of the extracellular matrix (O'Grady et al., 1998). However, our understanding of the functional relevance of such RPTP interactions with non-soluble ligands is very limited, perhaps because of inadequacy of the available assays. In vitro rescue of TCR signaling by CD45 occurs efficiently in the absence of the CD45 ectodomain (Trowbridge and Thomas, 1994), and yet the diversity generated by the intricate splicing pattern affecting the ectodomain is clearly crucial to generating an appropriate T-cell repertoire and homing behavior in vivo (Kozieradzki et al., 1997). As opposed to this plethora of non-soluble ligands, instances of soluble ligands are thus far limited to the high-affinity interaction of RPTP $\beta$ / $\zeta$  with the cytokines pleiotrophin and midkine (Maeda et al., 1999; Maeda and Noda, 1998), which was recently shown to inhibit RPTP $\beta$ / $\zeta$  activity (Meng et al., 2000; see below).

How might ligand association affect RPTP activity? Two non-exclusive mechanisms can be readily envisioned. The first depends on the topological considerations regarding access of RPTPs to their substrates discussed above. In this model, ectodomain-dependent interactions are mainly of 'architectural' importance, mediating recruitment and assembly into a cellular signaling complex or membrane microdomain. The second model relies on ligand binding intrinsically affecting RPTP activity through changes in oligomerization. Dimerization underlies ligand-dependent regulation of many receptor types (Weiss and Schlessinger, 1998), and the well-documented regulatory consequences of cross-linking CD45 (using antibodies (Trowbridge and Thomas, 1994) or chimeric proteins (Desai et al., 1993)) early on provided support for the possible regulatory significance of RPTP clustering.

In the case of receptor tyrosine kinases, ligand-induced juxtaposition favors transphosphorylation of the activation loops (perhaps in combination with conformational effects; Jiang and Hunter, 1999). This in turn boosts intrinsic catalytic activity and generates phosphorylation-based docking sites that enhance the affinity of the kinase for substrates or effectors (Weiss and Schlessinger, 1998). Although transphosphorylation-based activation clearly cannot apply to

RPTPs, alternative mechanisms can be envisaged. The intracellular domains of some RPTPs undergo tyrosine phosphorylation, and there is good evidence that this modulates RPTP function (Autero et al., 1994; den Hertog et al., 1994; Lammers et al., 2000; Su et al., 1996; Zheng et al., 2000; see above). Hence, a possibility that remains to be explored is that clustering could result in changes in RPTP function by favoring transdephosphorylation between tyrosine-phosphorylated RPTPs.

An alternative, conformational, link between dimerization and RPTP activity has been proposed on structural grounds. All PTPs share a common tertiary structure and catalytic mechanism. Central to the PTP reaction is a phosphate-binding active-site pocket, the base of which contains a nucleophilically acting cysteine residue. Following breakage of the phosphate bond in the substrate, this residue partakes in formation of a transient cysteinyl-phosphate intermediate. A conserved aspartate, appropriately positioned on a separate loop, subsequently acts as a general acid/base catalyst for the leaving tyrosine-containing substrate and for regeneration of the active enzyme from the intermediate (reviewed by Denu and Dixon, 1998). This fold and mechanism also apply to RPTPs (Hoffmann et al., 1997; Nam et al., 1999), but a surprising variation on this theme has been observed for RPTP $\alpha$  (Bilwes et al., 1996). The D1 domain of this RPTP crystallizes as a dimer, in which the interface surface area is in the range typical for high-affinity interactions. More particularly, a structurally constrained N-terminal helix-turn-helix 'wedge' inserts itself into the active site of the other monomer in the dyad, causing a mutual blocking of substrate access and interference with juxtaposition of the aspartate-containing loop. If this dimeric D1 structure forms *in vivo*, we can predict from our knowledge of the PTP reaction mechanism that dimeric RPTP $\alpha$  is catalytically inactive.

This predicted negative effect of dimerization has received some remarkable preliminary *in vivo* support in the cases of CD45 and RPTP $\alpha$ . Forced homodimerization of mutant forms of these RPTPs results in loss of function (assayed by reconstitution of TCR signaling (Desai et al., 1993) or rescue of normal SRC kinase activity (Jiang et al., 1999)). Strikingly, mutation of the wedge motif in each case reversed homodimerization-induced inhibition, which suggests that the wedge is instrumental in the inhibitory effect of dimerization (Jiang et al., 1999; Majeti et al., 1998). *In vivo* cross-linking and fluorescence-resonance-energy-transfer experiments suggest that dimerization of RPTP $\alpha$  can be extensive; however, it appears to be mediated not solely by D1-wedge interactions but also by the transmembrane and ectodomains (G. Jiang and T. Hunter; L. G. J. Tertoolen, C. Blanchetot, and J. den Hertog; personal communication). Heterodimeric interactions might also occur between the wedge region of one RPTP and the D2 domain of another (Wallace et al., 1998). Perhaps heterodimeric interactions between different RPTPs might underlie some of the complex genetic interactions observed in *Drosophila* among members of the RPTP family (Desai et al., 1997).

In spite of these provocative experiments, a number of issues need to be resolved, and extensive further testing of this model is needed. First, the *in vivo* experiments with CD45 and RPTP $\alpha$  have relied on artificially induced dimers, whose conformation might deviate from the physiological

conformation (Jiang and Hunter, 1999). Second, equally extensive structural evidence for other RPTPs is at odds with the model. Thus, whereas D1 of RPTP $\mu$  also crystallizes as a dimer, the relative orientation of RPTP $\mu$  D1 monomers is such that the (structurally similar) wedge would not interfere with substrate access (Hoffmann et al., 1997). Similarly, the joint D1-D2 structure of LAR predicts that D2 would sterically interfere with the close juxtaposition of D1 domains necessary to enable the wedge to block substrate access. The rigidity of the linker region and the significant extent of the buried D1-D2 surface area would impose a rigid orientation of both domains with respect to each other (Nam et al., 1999). Hence, inhibitory dimerization might be more relevant for RPTP $\alpha$  or CD45 than for other RPTPs such as PTP $\mu$  or LAR. Third, no direct links between binding of ligands and changes in dimerization, or between dimerization status and intrinsic enzymatic activity, have been documented. Conceivably, ligands (*cis* or *trans*) could act either as RPTP activators, if they have monomerizing properties, or as inhibitors, if they have dimerization-inducing properties. A striking and recent observation in this respect is the inhibitory effect of pleiotrophin on the intrinsic phosphatase activity of its receptor, RPTP $\beta/\zeta$ , which might account for the stimulatory effect of pleiotrophin on tyrosine phosphorylation of  $\beta$ -catenin (Meng et al., 2000). This first instance of *in vivo* regulation of an RPTP by a physiological and soluble ligand should now allow efficient further analysis of the potential regulatory relevance of dimerization and of D1-wedge interactions.

## PERSPECTIVES

Our understanding of the contributions of RPTPs to cell signaling has been evolving – yet remains spotty. Perhaps, some themes touched upon here – the combination of positive and negative functions within the same molecule, the contribution of multiple PTPs to control of a single pathway – will be confirmed through further analysis. If so, it is tempting to speculate that they constitute evolutionary safeguards against the tumorigenic consequences that could be associated with somatic loss of negatively acting PTPs.

Recent developments are increasingly focusing on critical functions of RPTPs in adhesion-dependent spreading and migratory events (Table 1; Angers-Loustau et al., 1999b; Maeda and Noda, 1998; Muller et al., 1999) and in neurite outgrowth (Arregui et al., 2000; Burden-Gulley and Brady-Kalnay, 1999; Gershon et al., 1998; Ledig et al., 1999; Stoker and Dutta, 1998). The broad involvement of PTPs in these processes might reflect their particular suitability to mediating dynamic remodeling events that rely on active turnover of cellular structures, and on recurrent cycles of making and unmaking of connections between a cell and its environment.

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## Note added in proof

A potentially additional target for regulation of membrane-

associated SFKs by PTPs, the Csk binding protein Cbp, was recently described by Kawabuchi et al. (2000) *Nature* **404**, 999-1003. Improved insights into the role of *Drosophila* DLAR in growth cone motility, derived from genetic analysis, are summarized by Lanier and Gertler (2000) *Curr. Opin. Neurobiol.* **10**, 80-87.

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